NON-INVASIVE MONITORING OF PHARMACOKINETICS AND PHARMACODYNAMICS FOR PHARMACOLOGICAL DRUG PROFILING IN CHILDREN AND ADOLESCENTS

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CHAPTER 1

Introduction

Until approximately 15 years ago, pharmaceutical companies rarely considered the needs of children when developing medicines, even though the majority of active substances licensed by the European Agency for the Evaluation of Medicinal Products (EMA) were considered to be relevant for use in children. Thus, fewer than half of all licensed active substances considered relevant for use in children had a pediatric indication in at least a subset of the pediatric population[1]; moreover, the off-label or unlicensed use of drugs in pediatric pharmacotherapy was highly prevalent among many European countries[2–4]. Although off-label use does not necessarily reflect off-knowledge use[5], off-label use can be a frequent cause of adverse events among hospitalized children[6]. In addition, the availability of several classes of medicines with a pediatric indication was limited, as pediatric medicines may not be age-appropriate (for example, they may not be dose-applicable or come in a suitable formulation), even if their use is authorized[7]. These issues are pertinent to pediatric pharmacology in general[8], but they may be even more pertinent to children and adolescents with a psychiatric[9] or neurological disorder (such as epilepsy[10] or pain[11]).

DIFFERENCES BETWEEN CHILDREN AND ADULTS WITH CNS DISEASE

The pharmacological treatment of children and adolescents with psychiatric disorders, as well as several neurological disorders, has traditionally followed the development program in adults, without cross-age validation or recognizing the inherent differences between children and adults with respect to their neuropsychopathology and neuropsychopharmacology[12,13]. For example, unlike in adulthood, the manifestation of schizophrenia in childhood and adolescence carries a particularly poor prognosis, and this poor outcome may be due to the disease's effect during a highly susceptible time in the development and neurobiological maturation of children and adolescents[14]. Moreover, in contrast to adults who experience seizures, many newborn infants and children who experience seizures remain refractory to therapy[15]. Finally, some disorders such as certain epilepsy syndromes are specific to children and have therefore been largely neglected, particularly with respect to drug development.

In addition, because of development-specific differences in pharmacokinetics (PK) and/or pharmacodynamics (PD), the relationships between drug action and drug exposure in children cannot be understood fully by simply extrapolating information from adult patients. For example, PK can differ considerably between children and adults as a result of physiological differences, differences in the maturity of enzyme systems, and differences in clearance mechanisms[16]. Age-related differences in PK can lead to differences in the blood concentration of a drug even when the same dose is given to a child and an adult, or to a young child and an older child, as reported for methylphenidate[17]. The brain-to-serum concentration ratio of a drug can also vary with age, potentially leading to age-dependent differences in concentration at the drug's site of action. For example, children with bipolar disorder have been reported to have lower lithium brain-to-serum ratios than adults[18]. In addition, several aspects of drug therapy may be related to brain development. Neural development in childhood and adolescence involves a highly coordinated sequence of events that is characterized by both progressive and regressive processes. As a result of this sequence of events, the development of gray matter, white matter, total brain volume, and neuronal connectivity is age-dependent[19], and perturbations in these developmental patterns may play a central role in the pathogenesis of several childhood neuropsychiatric and neurodevelopmental disorders, including autism, ADHD, fragile X syndrome, 22q11 deletion syndrome, Williams syndrome, Down syndrome, and Turner syndrome[20]. On the other hand, far less is known regarding the changes in brain function that result from these age-dependent and disorder-related changes in brain structure. However, a growing body of data suggests that healthy children have differential trends in the development of task performance in distinct functional areas of the central nervous system (CNS)[21–24], and children with neuropsychiatric disorders may have impaired neuropsychological function in several domains, as reported for ADHD[25]. Brain development can also affect drug efficacy and pharmacoresistance[13]. For example, monoaminergic systems, the target for most CNS drugs, undergo considerable plasticity and rearrangement during childhood and

adolescence[26–28], and animal studies have revealed age-related differences in the drug responses of several psychostimulant drugs such as amphetamine[29], cocaine[30,31], and methylphenidate[32,33]. In addition, γ-aminobutyric acid (GABA)—the activity of which is affected in many neurological and neurodevelopmental disorders[34] – excites immature neurons but inhibits neurons in the normal adult brain[35]. As a result of these age-dependent differences in PK and PD, children may respond differently than adults to CNS drugs. Unfortunately, our understanding of age-dependent differences in PK and PD is based almost entirely on animal models, and remarkably few clinical trials in humans have focused on understanding the age-dependent differences in response to CNS drugs.

DRUG DEVELOPMENT FOR CHILDREN WITH CNS DISEASE

In addition, the registration of CNS drugs for use in children and adolescents has lagged behind new developments in adults[36]. In particular, the steps involved in recognizing, classifying, and treating psychiatric disorders generally proceed much more slowly in children and adolescents than in adults[12]. In fact, the field of clinical psychopharmacology has only recently begun to include children and adolescents on a relatively large scale. This delay is the result of a combination of factors, including concerns regarding drug safety and tolerance in this vulnerable population, diagnostic uncertainties, and the pharmaceutical industry's reluctance to seek pediatric registration and to perform labeling studies for diagnoses that are not traditionally considered to be predominant disorders of childhood and adolescence (for example schizophrenia and bipolar disorder)[37]. In terms of clinical research, several areas within the field of pediatric neuropsychopharmacology have been neglected, including—but not limited to—eating disorders, early-onset schizophrenia, mental retardation[38], and pediatric critical care settings[39]. With respect to epilepsy, the most prevalent severe neurological disease among children, extremely few trials have been performed in children compared to adults; moreover, most antiepileptic drug trials in children are performed only once, whereas the majority of adult trials are replicated[13]. The developmental aspects of judgment and decision-making in children and adolescents with neuropsychiatric disorders such as cognitive immaturity and impaired cognitive processing are also unique to pediatric neuropsychopharmacology research[40]. Unlike other fields within pediatric pharmacology (such as pediatric oncology), child and adolescent psychiatry and pediatric neurology have not been tightly integrated into psychopharmacological networks, and professional societies for child and adolescent psychiatry have only recently begun to establish their own networks for psychopharmacology research[41,42].

The situation has been complicated even further by a decrease in the number of new drug registrations for psychiatric and neurological indications in adults. Such research is generally considered to be too risky, as the subjective nature of endpoints in psychiatry and neurology makes it difficult to determine whether a drug is effective, even using large-scale trials. As a result, the major changes in CNS drugs over the years have focused primarily on pharmacological modifications (such as improving the drug's intrinsic efficacy, selectivity, and/or kinetic properties[43]) rather than developing novel treatments.

Despite the relative paucity of scientific data to support the safe and efficacious use of CNS drugs in children and adolescents, and despite a lack of novel treatments, the number of treated children and adolescents, as well as the duration of exposure to CNS drugs, has increased substantially over the past few decades[44–46]. To close this gap, researchers need to acquire evidence regarding the adequate dosing, efficacy, and safety of these treatments[47]. Recent European legislation (the EU Pediatric Regulation) will likely drive an increase in pediatric trials and specific label changes, dosing recommendations, and age-appropriate formulations. Unless a specific waiver or deferral is granted, the Regulation requires the industry to plan clinical trials in children early in the development of new medicines for treating adults or for line extensions for on-patent medications. Several challenges have emerged when working within the framework of this new legislation[48]. For example, performing randomized, controlled trials in children can raise specific technical, logistic, legal, and financial difficulties that are not usually associated with trials performed in adults. In addition, even studies that are performed

successfully in children are not necessarily maximally informative, as many studies measure PK only or perform a single post-dose PD measurement. Because of these challenges—even in fields with extensive pediatric research such as ADHD—it can be difficult to reach valuable and relevant conclusions regarding pharmacological profiles and/or dose-dependent effects based solely on currently available studies.

NEED FOR NON-INVASIVE MEASUREMENT METHODS

Therefore, there is an urgent need for validated assessment tools that are suitable for evaluating the efficacy and safety of CNS drugs in the pediatric population[49]. In addition, because clinical research in pediatric patients is generally hampered by interrelated logistic and ethical constraints (including the limited number, scope, and invasiveness of study-related interventions that can be performed if they fall outside the realm of routine clinical care), researchers should attempt to reduce the burden placed on participating children and adolescents by using non-invasive or minimally invasive measurement methods. Changes to these methods that are designed to reduce patient burden (for example, implementing a change in the sampling procedure) have been reported to increase patient enrollment in studies of rare pediatric diseases[48].

The most accessible and non-invasive means to measure drug activity in the brain is to measure drug-related CNS functional activity using methods that provide sufficient sensitivity and specificity[43]. Although validated biomarkers are rare in neurology and psychiatry, many neuropsychiatric drugs affect a variety of CNS functions in a dose-dependent and concentration-dependent manner, particularly drugs that affect neurotransmitter activity. This provides the opportunity to quantify CNS effects, even in situations in which the activity measured is not an essential step in the pathogenic cascade. The affected functions can often be roughly or partially linked to the specific pharmacological mechanism (for example, linking $GABA_A$-ergic activation to reduced saccadic peak velocity). Thus, these physiological functions can be useful biomarkers for measuring a pharmacological effect, even if they have no clear functional relationship with psychosis, anxiety, or depression. It is very likely that a clear concentration-dependent effect of a highly specific compound is mediated by the drug's mechanism of action, even if the functional relationship itself is not clear[43], thereby enabling researchers to compare different drug formulations and age-dependent differences in drug profiles.

To relate drug-related changes in CNS functional activity to changes in PK, drug concentrations must be measured. Traditional PK protocols—with multiple samples and indwelling catheters or multiple venipunctures—are undesirable in therapeutic pediatric drug research, and they are strictly unacceptable in non-therapeutic pediatric drug research. To overcome some of these limitations, other sample collection methods for determining drug concentration (for example, saliva sampling) have been developed and validated. Studies have reported that patients and parents prefer saliva sampling over venous blood drawing[50], and some drugs can be detected in the saliva relatively soon after administration, enabling the researcher to compile a concentration-time profile. This method has the added benefit of allowing on-site testing without the need for medical personnel or complicated post-collection sample processing, thereby further decreasing the burden placed on the children.

A drug's secretion and distribution in the saliva is dependent upon its physico-chemical properties. Lipophilicity, degree of ionization, and protein binding are the major determinants of the saliva:plasma concentration (S/P) ratio[51]. For example, drugs that are heavily protein bound and drugs that are extremely hydrophilic and positively charged at physiological pH (for example, aminoglycosides) can be undetectable in saliva. The transition of drugs to the CNS is favored by low molecular weight, a lack of ionization at physiological pH, and lipophilicity[52], all of which theoretically favor the drug's secretion into the saliva. In certain cases, saliva concentration may even be preferred over total plasma concentration, particularly for highly protein-bound drugs, as saliva concentration can reflect the free fraction of a non-ionized drug (and thus reflects the intracellular concentration in target tissues). For example, the concentration of phenytoin in cerebrospinal fluid correlates more closely

with the saliva concentration than the blood concentration[53]. Saliva sampling is also recommended over plasma sampling for the therapeutic monitoring of several other anti-epileptic drugs[54]. Unfortunately, however, the usefulness of determining the saliva concentration of several other drugs has been questioned because of variability in the S/P ratio. Interpreting the saliva drug concentration of neutral, weakly acidic (pK_a > 8.5), or weakly basic (pK_a < 5.5) drugs with negligible protein binding (such as ethanol, antipyrine, and paracetamol) is relatively easy, as the S/P ratio is approximately 1. In contrast, interpreting the saliva drug concentration of drugs with other properties can be challenging, as the S/P ratio can be low for acidic drugs (pK_a < 8.5) and drugs with high protein binding (such as caffeine and phenobarbital), and the concentration can be influenced by active transport mechanisms (such as lithium) or the occurrence of an alkaline reaction in aqueous solutions, for example in the case of basic drugs (pK_a > 5.5) with low protein binding (such as methylphenidate, procainamide, amphetamine, and lidocaine). Therefore, the use of saliva sampling for measuring concentration of several drug types has been limited. In addition, because many drugs are administered orally, buccal contamination can affect the S/P ratio at early time points after administration. Nevertheless, if the sources of variability in the S/P ratio can be minimized or quantified, measuring the saliva drug concentration might be a meaningful alternative to measuring plasma drug concentration, particularly for the aforementioned drug types.

SCOPE OF THIS THESIS

In this thesis, we explored non-invasive methods for monitoring the pharmacokinetics and pharmacodynamics of commonly used CNS stimulants (methylphenidate and caffeine) and depressants (ethanol and melatonin) in children and/or adolescents. Neuropsychological and neurophysiological functions were measured longitudinally using the NeuroCart, a battery of tests developed at the Centre for Human Drug Research that includes tests for alertness, visuomotor coordination, motor control, memory, and subjective drug effects. Drug concentrations in children and adolescents were measured non-invasively. We evaluated the feasibility of using saliva as an alternative to plasma for measuring drug concentration in two studies. For this purpose, caffeine was chosen as an example of a basic CNS drug that is primarily non-ionized in human saliva, and methylphenidate was chosen as a typical basic CNS drug that is primarily ionized in human saliva. This thesis concludes with the report of two clinical trials that were designed to develop age-appropriate formulations for potential use in children.

In **chapter 2**, the impact of the Pediatric Regulation on the development of pediatric drugs – including CNS drugs – is evaluated. In this study, we evaluated for which drug classes and therapeutic subgroups pediatric development was agreed by (or waived by) the Regulation. In addition, we evaluated whether the Regulation is likely to increase the development of drug classes for pediatric conditions for which there exists an unmet pediatric need based on usage and availability data.

The psychostimulant methylphenidate (MPH) is the most commonly prescribed medication for treating pediatric attention-deficit/hyperactivity disorder (ADHD). However, previously published studies investigating the effects of immediate-release methylphenidate (MPH-IR) have yielded contradictory results due to several sources of variability, including a lack of standardized biomarkers and drug-effect measurements. Despite its widespread use, and despite extensive research, researchers still lack useful, validated biomarkers for studying the effect of MPH in children with ADHD. In **chapter 3,** we identified generally applicable, non-invasive biomarkers for monitoring the acute effects of MPH-IR in children and adolescents with ADHD. The presence of unexplained variations in the S/P ratio during the time course of both MPH-IR and extended-release MPH can interfere with the further exploration of saliva as an alternative method for determining MPH concentration in children. Therefore, a liquid chromatography-tandem mass spectrometric method that uses a hydrophilic interaction liquid chromatography column (HILIC) was validated and is presented in **chapter 4**. This analytical tool provides an accurate and precise quantification of MPH in both plasma and saliva samples. **Chapter 5** describes a study in which we attempted to

identify sources of variability in MPH concentration and to determine the correlation between MPH concentration in the saliva and plasma in healthy adult volunteers using a population-based PK approach.

Chapter 6 describes a study of the effect profile of low-dose caffeine in healthy adolescents. Caffeine concentration was measured non-invasively in saliva samples. Because saliva can be contaminated by residual caffeine after drinking a caffeinated beverage, a second study was performed in young adult subjects in order to measure contamination after drinking a caffeinated beverage compared to swallowing a caffeine capsule. In addition, previous studies in adults related caffeine effects to changes in plasma concentration; therefore, simultaneous saliva and plasma samples were collected, and the S/P ratio of caffeine was measured. Based on the data obtained from this kinetics study, a population-based PK model was built in order to obtain estimated plasma drug levels in adolescents; this model could prove useful in the development of a caffeine pharmacokinetics-pharmacodynamics (PK/PD) model in adolescents.

Chapter 7 describes a study to measure the acute effects of low-dose alcohol on sensitive biomarkers for alcohol effects in healthy adolescents. To correlate the measured effects with concentration, alcohol concentration was measured non-invasively using end-expired breath sampling. A PK/PD model was then developed using data from this study with adolescents as well as data collected from previous alcohol studies performed in adults in order to characterize alcohol PK, the effects on objective and subjective biomarkers, and sources of variability in PK and PD, including age.

Sleep problems are highly prevalent among children with neurodevelopmental disorders and can have a substantial impact on the child and the child's family. Melatonin is effective for treating sleep problems in these children, and melatonin appears to have a favorable short-term and long-term risk profile, which has led to the increasingly widespread clinical use of melatonin as an off-label medicine. However, no commercially available, age-appropriate prolonged-release melatonin preparation is currently available. Melatonin was identified by the EMA as having a pediatric therapeutic need, including the need to develop an age-appropriate sustained-release formulation and the need to collect data regarding melatonin's PK, efficacy, and safety in children with autism and a sleep disorder. The study described in **chapter 8** is part of a Pediatric Investigation Plan (PIP) under the Pediatric Regulation and was a cross-over ascending dose study of Circadin (1 mg mini-tablets), a prolonged-release melatonin formulation. In this study, the PK profile, safety, and acceptability of Circadin mini-tablets were evaluated in 16 children and adolescents with autism and a sleep disorder. Whole-saliva samples were collected non-invasively from passive drool, and melatonin concentration was measured.

Because of its rapid onset and rapid recovery profile, midazolam is the medication of choice for providing conscious sedation and management of epileptic seizures. Nasal delivery of midazolam is a patient-friendly alternative to parenteral delivery routes. Nasal delivery offers several practical advantages; for example, it allows for direct, easy, needle-free administration, and it can be administered safely without professional assistance. However, previous formulations for delivering midazolam nasally have not been very successful due to the lack of solvents that can dissolve midazolam at therapeutic dosages without causing nasal mucosa damage. Therefore, in **chapter 9**, we evaluated the PK, efficacy, and tolerability of a new, highly concentrated aqueous midazolam formulation (Nazolam) in healthy adult volunteers. This new formulation has potential for use in providing conscious sedation and epilepsy management in children and adolescents.

In **chapter 10**, the feasibility and applicability of saliva sampling in pediatric populations will be discussed based on data obtained from pediatric clinical studies of caffeine, methylphenidate, and melatonin described in this thesis. In addition, the (potential of) pharmacological profiling of pharmacodynamics in children and adolescents will be discussed based on literature review and clinical studies of methylphenidate, caffeine, alcohol and midazolam. This chapter concludes with potential practical applications of this approach and suggestions for future directions.

REFERENCES

1 Ceci A, Felisi M, Baiardi P, Bonifazi F, Catapano M, Giaquinto C et al. Medicines for children licensed by the European Medicines Agency (EMEA): the balance after 10 years. *Eur J Clin Pharmacol* 2006; 62(11):947-952.

2 Conroy S, Choonara I, Impicciatore P, Mohn A, Arnell H, Rane A, et al. Survey of unlicensed and off label drug use in paediatric wards in European countries. European Network for Drug Investigation in Children. BMJ. 2000;320:79-82.

3 Pandolfini C, Bonati M. A literature review on off-label drug use in children. *Eur J Pediatr.* 2005;164:552-558.

4 Cuzzolin L, Atzei A, Fanos V. Off-label and unlicensed prescribing for newborns and children in different settings: a review of the literature and a consideration about drug safety. *Expert Opin Drug Saf.* 2006;5:703-718.

5 Rose K. European Union Pediatric Legislation Jeopardizes Worldwide, Timely Future Advances in the Care of Children With Cancer. *Clin Ther.* 2014;36:163-177.

6 Mason J, Pirmohamed M, Nunn T. Off-label and unlicensed medicine use and adverse drug reactions in children: a narrative review of the literature. *Eur J Clin Pharmacol.* 2012;68:21-28.

7 van Riet-Nales DA, de Jager KE, Schobben AF, Egberts TC, Rademaker CM. The availability and age-appropriateness of medicines authorized for children in The Netherlands. *Br J Clin Pharmacol.* 2011;72:465-473.

8 Roberts R, Rodriguez W, Murphy D, Crescenzi T. Pediatric drug labeling: improving the safety and efficacy of pediatric therapies. JAMA. 2003;290:905-911.

9 Radley DC, Finkelstein SN, Stafford RS. Off-label prescribing among office-based physicians. *Arch Intern Med.* 2006;166:1021-1026.

10 Chiron C, Kassai B, Dulac O, Pons G, Nabbout R. A revisited strategy for antiepileptic drug development in children: designing an initial exploratory step. CNS *Drugs.* 2013;27:185-195.

11 Davies EH, Ollivier CM, Saint RA. Paediatric investigation plans for pain: painfully slow! *Eur J Clin Pharmacol.* 2010;66:1091-1097.

12 Stoyanova-Beninska VV, Wohlfarth T, Isaac M, Kalverdijk LJ, van den Berg H, Gispen-de WC. The EU paediatric regulation: effects on paediatric psychopharmacology in Europe. *Eur Neuropsychopharmacol.* 2011;21:565-570.

13 Chiron C, Dulac O, Pons G. Antiepileptic drug development in children: considerations for a revisited strategy. *Drugs.* 2008;68:17-25.

14 Clemmensen L, Vernal DL, Steinhausen HC. A systematic review of the long-term outcome of early onset schizophrenia. BMC *Psychiatry.* 2012;12:150.

15 Noam Y, Raol YH, Holmes GL. Searching for new targets for treatment of pediatric epilepsy. *Epilepsy Behav.* 2013;26:253-260.

16 Ginsberg G, Hattis D, Sonawane B, Russ A, Banati P, Kozlak M, et al. Evaluation of child/adult pharmacokinetic differences from a database derived from the therapeutic drug literature. *Toxicol Sci.* 2002;66:185-200.

17 Wigal SB, Gupta S, Greenhill L, Posner K, Lerner M, Steinhoff K, et al. Pharmacokinetics of methylphenidate in preschoolers with attention-deficit/hyperactivity disorder. *J Child Adolesc Psychopharmacol.* 2007;17:153-164.

18 Moore CM, Demopulos CM, Henry ME, Steingard RJ, Zamvil L, Katic A, et al. Brain-to-serum lithium ratio and age: an in vivo magnetic resonance spectroscopy study. *Am J Psychiatry.* 2002;159:1240-1242.

19 Toga AW, Thompson PM, Sowell ER. Mapping brain maturation. *Trends Neurosci.* 2006;29:148-159.

20 Dennis EL, Thompson PM. Typical and atypical brain development: a review of neuroimaging studies. *Dialogues Clin Neurosci.* 2013;15:359-384.

21 Brocki KC, Bohlin G. Executive functions in children aged 6 to 13: a dimensional and developmental study. *Dev Neuropsychol.* 2004;26:571-593.

22 Luciana M, Nelson CA. The functional emergence of prefrontally-guided working memory systems in four- to eight-year-old children. *Neuropsychologia.* 1998;36:273-293.

23 van den Wildenberg WP, van der Molen MW. Developmental trends in simple and selective inhibition of compatible and incompatible responses. *J Exp Child Psychol.* 2004;87:201-220.

24 Karatekin C. Development of attentional allocation in the dual task paradigm. *Int J Psychophysiol.* 2004;52:7-21.

25 Chamberlain SR, Robbins TW, Winder-Rhodes S, Muller U, Sahakian BJ, Blackwell AD, et al. Translational approaches to frontostriatal dysfunction in attention-deficit/hyperactivity disorder using a computerized neuropsychological battery. *Biol Psychiatry.* 2011;69:1192-1203.

26 Gelbard HA, Teicher MH, Faedda G, Baldessarini RJ. Postnatal development of dopamine D1 and D2 receptor sites in rat striatum. *Brain Res Dev Brain Res.* 1989;49:123-130.

27 Court JA, Perry EK, Johnson M, Piggott MA, Kerwin JA, Perry RH, et al. Regional patterns of cholinergic and glutamate activity in the developing and aging human brain. *Brain Res Dev Brain Res.* 1993;74:73-82.

28 Chen YI, Choi JK, Xu H, Ren J, Andersen SL, Jenkins BG. Pharmacologic neuroimaging of the ontogeny of dopamine receptor function. *Dev Neurosci.* 2010;32:125-138.

29 Bolanos CA, Glatt SJ, Jackson D. Subsensitivity to dopaminergic drugs in periadolescent rats: a behavioral and neurochemical analysis. *Brain Res Dev Brain Res.* 1998;111:25-33.

30 Laviola G, Wood RD, Kuhn C, Francis R, Spear LP. Cocaine sensitization in periadolescent and adult rats. *J Pharmacol Exp Ther.* 1995;275:345-357.

31 Chen YI, Choi JK, Xu H, Ren J, Andersen SL, Jenkins BG. Pharmacologic neuroimaging of the ontogeny of dopamine receptor function. *Dev Neurosci.* 2010;32:125-138.

32 Levant B, Zarcone TJ, Davis PF, Ozias MK, Fowler SC. Differences in methylphenidate dose response between periadolescent and adult rats in the familiar arena-novel alcove task. *J Pharmacol Exp Ther.* 2011;337:83-91.

33 Chen YI, Choi JK, Xu H, Ren J, Andersen SL, Jenkins BG. Pharmacologic neuroimaging of the ontogeny of dopamine receptor function. *Dev Neurosci.* 2010;32:125-138.

34 Deidda G, Bozarth IF, Cancedda L. Modulation of GABAergic transmission in development and neurodevelopmental disorders: investigating physiology and pathology to gain therapeutic perspectives. *Front Cell Neurosci.* 2014;8:119.

35 Khazipov R, Khalilov I, Tyzio R, Morozova E, Ben-Ari Y, Holmes GL. Developmental changes in GABAergic actions and seizure susceptibility in the rat hippocampus. *Eur J Neurosci.* 2004;19:590-600.

36 Weissman MM, Klerman GL. Depression: current understanding and changing trends. *Annu Rev Public Health.* 1992;13:319-339.

37 Correll CU. Clinical psychopharmacology of pediatric mood stabilizer and antipsychotic treatment, part 1: challenges and developments. *J Clin Psychiatry.* 2007;68:1301-1302.

38 Tobi H, Scheers T, Netjes KA, Mulder EJ, de BA, Minderaa RB. Drug utilisation by children and adolescents with mental retardation: a population study. *Eur J Clin Pharmacol.* 2005;61:297-302.

39 Stoddard FJ, Usher CT, Abrams AN. Psychopharmacology in pediatric critical care. *Child Adolesc Psychiatr Clin N Am.* 2006;15:611-655.

40 Spetie L, Arnold LE. Ethical issues in child psychopharmacology research and practice: emphasis on preschoolers. *Psychopharmacology (Berl).* 2007;191:15-26.

41 Kolch M, Schnoor K, Fegert JM. The EU-regulation on medicinal products for paediatric use: impacts on child and

adolescent psychiatry and clinical research with minors. *Eur Child Adolesc Psychiatry.* 2007;16:229-235.
42 Vitiello B. Research in child and adolescent psychopharmacology: recent accomplishments and new challenges. *Psychopharmacology (Berl).* 2007;191:5-13.
43 van GJ, Cohen A. Vanishing clinical psychopharmacology. *Br J Clin Pharmacol.* 2011;72:1-5.
44 Kalverdijk LJ, Tobi H, van den Berg PB, Buiskool J, Wagenaar L, Minderaa RB, et al. Use of antipsychotic drugs among Dutch youths between 1997 and 2005. *Psychiatr Serv.* 2008;59:554-560.
45 Schirm E, Tobi H, Zito JM, de Jong-van den Berg LT. Psychotropic medication in children: a study from the Netherlands. *Pediatrics.* 2001;108:E25.
46 Vitiello B. Pharmacoepidemiology and pediatric psychopharmacology research. *J Child Adolesc Psychopharmacol.* 2005;15:10-11.
47 Wohlfarth T, Kalverdijk L, Rademaker C, Schothorst P, Minderaa R, Gispen-de WC. Psychopharmacology for children: from off label use to registration. *Eur Neuropsychopharmacol.* 2009;19:603-608.
48 Winzenburg G. More than 5 years of European Paediatric Regulation: Statistics and industrial experience. *Int J Pharm.* 2014.
49 Stoyanova-Beninska VV, Wohlfarth T, Isaac M, Kalverdijk LJ, van den Berg H, Gispen-de WC. The EU paediatric regulation: effects on paediatric psychopharmacology in Europe. *Eur Neuropsychopharmacol.* 2011;21:565-570.
50 Gorodischer R, Burtin P, Hwang P, Levine M, Koren G. Saliva versus blood sampling for therapeutic drug monitoring in children: patient and parental preferences and an economic analysis. *Ther Drug Monit.* 1994;16:437-443.
51 Haeckel R, Hanecke P. Application of saliva for drug monitoring. An in vivo model for transmembrane transport. *Eur J Clin Chem Clin Biochem.* 1996;34:171-191.
52 Pardridge WM. Recent advances in blood-brain barrier transport. *Annu Rev Pharmacol Toxicol.* 1988;28:25-39.
53 Troupin AS, Friel P. Anticonvulsant level in saliva, serum, and cerebrospinal fluid. *Epilepsia.* 1975;16:223-227.
54 Gorodischer R, Koren G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. *Dev Pharmacol Ther.* 1992;19:161-177.

CHAPTER 2

The European Pediatric Regulation: will it provide children with the medicines they need?

Submitted

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ABSTRACT

The EU Pediatric Regulation requires the pharmaceutical industry to plan clinical trials in children at an early development stage in adults. The aim of this study was to evaluate whether this initiative is likely to provide medicines that children need. We evaluated the drug classes for which pediatric development was either agreed for development or was waived by the EMA from 2007 until March 2012. We questioned if the scope of drug classes for which pediatric development was agreed reflects trends in Dutch pediatric usage and availability data, the relative distribution of drug classes included in the EMA Pediatric Needs Lists, or drug expenditure data. Dutch pediatricians were asked if they perceived these pediatric medicines as being necessary. Allergens were excluded from the analysis. Approximately two-thirds of the medicines were agreed for pediatric development; deferral was granted for 83% percentage of these medicines. The majority of medicines agreed for pediatric development belonged to the drug class antineoplastic and immunomodulatory drugs, anti-infectives for systemic use, and drugs that act upon the blood and blood-forming organs. The majority of agreed research and development occurred for drug classes for which drug expenditure is currently high (which reflected extensive adult use). For these drug classes, there appeared to be relatively little need for research and development based upon Dutch pediatric usage and availability data or based on the EMA Needs Lists. Dutch physicians working in pediatric healthcare were not convinced that medicines for which pediatric development was agreed were needed for clinical practice. Given the substantial public health investment and the potential negative effects of the Regulation on research in areas with bona fide needs, key improvements in the Regulation's implementation are recommended in order the ensure that the Regulation provides children with medicines they actually need.

Introduction

The Pediatric Regulation, introduced by the European Medicines Agency (EMA) in early 2007, changed the European regulatory environment for the development of pediatric medicines. One of the Regulation's strategies is to require industry to plan clinical trials in children in an early stage during drug development in adults or in case a new indication, formulation or administration route is investigated for adults for on-patent medicines. Marketing authorization applications for this type of medicines must contain the results of all previous studies and information described in the company's pediatric investigation plan (PIP) previously agreed by EMA's Pediatric Committee (PDCO). Prior to the Regulation's implementation, the lack of appropriate pediatric formulations in a large number of therapeutic areas[1] was an important reason for off-label and unlicensed drug use and was a major obstacle to the study of drugs in children. This situation was complicated further by a general lack of overlap between areas in which pediatric drug research was conducted and both pediatric therapeutic needs[2] and pediatric disease burden[3]. The Regulation – which is based upon unmet pediatric needs and establishes clear obligations and a system of incentives aimed at pharmaceutical companies – was expected to result in research more focused on children's needs and to drive fundamental changes in therapeutic options for treating pediatric patients[2]. In addition, the Regulation was expected to provide early access to newer, safer, more targeted treatments[4,5], and to lead to more options in terms of age-appropriate formulation types[6].

In July 2012, the EMA submitted a five-year interim report to the European Commission[7] in which they reflected on the experience acquired as a result of the Regulation. From 2008 through 2011, 13 new medicines, 30 new indications, and 9 new formulations of existing medicines were authorized for use in children based on PIPs that were agreed by the PDCO. Importantly, some pediatric therapeutic areas that predominantly affect children had been neglected in terms of pharmaceutical research[7]. However, the assessment of concordance between agreed pediatric drug development and pediatric needs was limited to determining the number of development plans by therapeutic area covered by the planned indication. In addition, some important issues were not considered in the report, including the Regulation's impact on the development of age-appropriate formulations, despite the specific emphasis placed upon this aspect by the Regulation.

Therefore, the objective of our study was to determine in further detail whether the initiative is likely to provide medicines that children need. First, we determined the drug classes and age groups for which pediatric development was either agreed or waived by the PDCO from 2007 until March 2012. We also evaluated whether the scope of drug classes for which pediatric development was agreed matched the trends in Dutch pediatric pharmaco-epidemiological prescription and availability data or drug expenditure data. Finally, we evaluated whether Dutch pediatricians believe that pediatric medicines developed and researched under the Regulation are actually needed.

Methods

Drug classes with agreed or waived pediatric development

A publicly available database on the EMA website[8] was searched in March 2012 for PDCO opinions and EMA decisions on company proposals for PIPs, waivers or modifications for the pediatric development of active substances. The Regulation includes a system for waiving medicines that are unlikely to benefit children and for deferring the start or completion of measures in the PIP until after authorization for adults in order to ensure that medicines are tested in children only when safe and to prevent unnecessary delays in the authorization for adults. After the PDCO has agreed to the PIP, the PIP can be modified by the company at a later stage by adding new knowledge or if the company encounters difficulties with its implementation that render the plan untenable or no longer appropriate. In these situations, the company may propose changes to the PIP or may request a deferral or waiver – based on

specific grounds from the PDCO. The types of EMA decisions that are posted on the EMA website therefore include decisions that (1) agree on a PIP proposed by the company, either with or without a partial waiver and/or deferral ('P'); (2) grant a waiver proposed by the company in all age groups for the listed condition or conditions ('W'); (3) are based on an application by the company for modifying an agreed PIP ('PM'); (4) refer to a refusal of a PIP proposed by the company ('RP'); (5) refer to a refusal of a waiver requested by the company in all age groups for the listed condition or conditions ('RW'); and (6) refer to a refusal of an application by the company to modify an agreed PIP ('RPM'). In our analysis, active substances with one or more PIPs with a decision type P, PM, or RPM were considered as active substances for which pediatric development was agreed by the EMA (referred to hereafter as 'medicines with agreed pediatric development'). Active substances with one or more PIPs with a decision type W were considered as active substances for which the EMA agreed to waive pediatric development (referred to hereafter as 'medicines with waived pediatric development').

DATA RETRIEVAL

Data were extracted using a pre-established study database (in a Microsoft Excel workbook) and included PDCO decision number, active substance, condition, therapeutic area, pharmaceutical form(s), route(s) of administration, company name, decision type, the date of the initial decision or last updated decision in the case of a request for modification, the age range of the pediatric population covered by the PIP, the type and number of required studies, waiver type, deferral (yes or no), invented name (if available) and the PIP's expected completion date. The pharmaceutical forms under each PIP were categorized into: peroral, topical/transdermal, ocular, nasal, parenteral, auricular, rectal, pulmonary, and vaginal. The oral pharmaceutical form was further subdivided in: solution/drops, emulsion/suspension, powder/multiparticulate, tablet, chewable tablet, and capsule. The pediatric subset was categorized into: younger than 2 years of age (infants and toddlers), 2-11 years of age (children), and 12-18 years of age (adolescents). If the researched age range included ages that fell within a specific age subgroup, this subgroup was listed as 'yes'.

ATC CODE ASSIGNMENT

Generic names of active substances in the study database were searched in a searchable version of the World Health Organization Collaborating Centers (WHOCC) database[9]. If the generic name was not included in the PIP, Thomson Reuters' Integrity database[10] was searched for this information. The WHO Anatomical Therapeutic Chemical (ATC) Classification anatomical main group and therapeutic subgroup retrieved from the WHOCC database (2013) were added to each active substance in the study database using a standardized approach. Medicinal products are classified according to the main therapeutic use of the main active ingredient, on the basic principle of only one ATC code for each route of administration. For active substances with only one entry in the WHOCC database, this unique ATC code was included in the study database. A medicinal product can be given more than one ATC code if it is available in two or more strengths or administration routes with clearly different therapeutic uses. Pharmaceutical forms for topical and systemic use are also given separate ATC codes. For active substances with multiple entries in the WHOCC database, planned pediatric indication and formulation were taken into account while choosing the most appropriate code. Combination products contain two or more active ingredients. Fixed combination products (e.g., amlodipine/valsartan) have a unique ATC code. For other ATC combination levels, not all active ingredients are searchable in the WHOCC database, but ATC code is based on the code of the main ingredient. Unique ATC codes for combination products in the study database were added if present. If no unique ATC code could be identified, general WHO guidelines were followed, i.e., different combination products sharing the same main active ingredient are usually given the same ATC, or the ATC code of the main ingredient was included (as the classification of combination products is decided by the main therapeutic use).

The ATC code for combination products and active substances with multiple entries in the WHOCC database were assigned based on the consensus of two researchers (authors A.D.M. and L.S.). For active substances that did not have an entry in the WHO database, the temporary ATC code given at the 2013 meeting of the WHO International Working Group for Drug Statistics Methodology[11]

was used if available. For the remaining substances, a 'fictitious' ATC code was assigned based on the ATC of a reference drug with the same target and indication (if available) or based on the drug target, therapeutic group, product class listed in the Thomson Reuters' Integrity database[10] (accessed in March 2014), or indication. For these active substances, an additional pharmacist (author R.R.) reviewed all of the collected data and the ATC proposed by the first two authors and provided input; based on this input, a final ATC was chosen.

DATA MERGING

Pediatric development was analyzed for each active substance. All of the data from various PIPs for a given active substance were merged, except for allergen products. PIPs for allergen products were left out of the analysis, as the information in the Decisions and Opinions was not specific enough to distinguish different active substances or pediatric development. For all other active substances, if a therapeutic area was addressed by multiple indications for a given active substance, it was considered only once in the analysis. If the same formulation type was considered in multiple PIPs, it was counted only once. If the pharmaceutical form in the PIP was intended for several administration routes, was constituted in several different ways, or if the pharmaceutical form was packaged in several different delivery devices, the pharmaceutical form was counted twice.

The EMA's online database includes several duplicate applications that share pediatric development. EMA decisions can be split or merged, and until 2010, duplicate applications could be submitted by a company for a given active substance that had—or planned to have—more than one marketing authorization and for which the scope of the development was the same (e.g., the same conditions, route of administration, and/or formulation). For example, one marketing authorization holder can have two or more authorizations in order to have different trade names, and two pharmaceutical companies can be joint holds of a global marketing authorization. The EMA database also holds 'temporal' duplicate applications (e.g., an EMA decision regarding a refused waiver for a particular active substance that is later followed by an agreed PIP for the same indication). Currently, duplicate PIPs are not identified on the EMA website. Because duplicate PIPs share a single pediatric development, all PIPs were checked manually for duplicate status (by reviewing the conditions, subset of the relevant pediatric population, administration route, formulation, and agreed studies). In the event of a duplicate application, only one PIP was considered in our analysis. In the event of a temporal duplicate (waiver followed by agreed PIP for same indication), only the EMA's most recent decision was included in the analysis. The input from the EMA was sought in case of unclarity regarding duplicate status.

DATA ASSESSMENT

All medicines for which pediatric development had been agreed and all active substances for which development was waived were listed according to drug classes and therapeutic subgroups. The number of formulation types and number of formulations were assessed by drug class.

Drug classes with an established or potential pediatric need

Data extracted from the EMA Pediatric Needs Lists[12] (published through July 2012) included the active substance, therapeutic area, authorized indication, and specified needs. Needs were categorized as 'formulation only' (research need for an age-appropriate or disease-appropriate formulation only), 'pediatric studies only' (only need to expand the indication to other indications or age groups, need for study, or definition of age limit), or 'full pediatric development' (the need for age-appropriate formulations and studies). Medicines for which there was only a need for the availability of the indication or age-appropriate formulation in all EU member states were not included in the analysis. Medicines were listed by drug class and evaluated for their specified needs type.

Trends in the prevalence of drug use in Dutch children

Data were obtained from the PHARMO Database Network, a population-based network of healthcare databases that combines data from various healthcare settings within the Netherlands. These data sources are linked on a patient level using validated algorithms[13–15]. For this study, drug dispensing data from 2005 through 2011 extracted from the PHARMO Out-patient Pharmacy Database were used. The Out-patient Pharmacy Database includes healthcare products prescribed by a general practitioner or specialist and dispensed by an out-patient pharmacy (coded according to the WHO ATC Classification System). The dispensing records include the type of product, date, strength, dosing regimen, quantity, route of administration, the prescriber's specialty, and the cost. Within each year from 2005 through 2011, a separate patient selection was performed; accordingly, we selected all children who were 0-18 years of age in a given calendar year and had at least one drug dispensed from the PHARMO out-patient pharmacy database (excluding vitamin K, as this is given to all children who are breast-fed)[16]. For each calendar year, age was assessed by subtracting the year of birth from the calendar year. Patients were grouped into specific age groups (see above). For each year in the study period, the number of children for whom any drug was dispensed was measured and then extrapolated to the general population of the Netherlands. Specifically, we multiplied the number of children counted by the number of inhabitants in the Netherlands; we then divided by the number of residents in the PHARMO catchment area (standardized for calendar year, age, and gender). Prevalence of use was reported per 10,000 children and was stratified by calendar year and age group. In addition, we counted and extrapolated the number of children for whom different anatomical classes of the ATC classification scheme (first level ATC code) were dispensed.

Trends in Dutch pharmaceutical expenditure

Drug volume consumed and drug prices for the years 2007 through 2011 were extracted by drug class from the GIP (*Genees- en hulpmiddelen Informatie Project*; in English: the Medicines and Aiding Devices Information Project) databank[17], an information system used by the Dutch Healthcare Insurance Board. Drug classes were listed by expenditure data (both overall and per user).

Survey of Dutch physicians working with children

All medicines for which a development plan was agreed by March 2012 were listed by EMA therapeutic area using an online survey that included information regarding agreed clinical studies in children. Fifty medical specialists and residents in the fields of pediatrics or child and adolescent psychiatry completed one or more surveys in which they were asked to indicate whether they need the medicine in their practice ('yes' or 'no'); if 'yes', would they (intend to) use the drug in their practice for treating the study population described in the clinical study and do they find the information obtained from the trials useful; if 'yes', would they prescribe the medicine.

Data analysis

Descriptive data were analyzed using Microsoft Excel 2007. Data from the PHARMO Database Network were analyzed using SAS programs organized within SAS Enterprise Guide version 4.3 (SAS Institute Inc., Cary, NC, USA) and conducted under UNIX using SAS version 9.2.

Results

Research and development under the Pediatric Regulation

765 EMA decisions were extracted from the EMA database, of which 117 decisions concerned allergens (which were excluded from the analysis). Of the remaining decisions, 9% were (temporal) duplicates. Thus, a total of 590 EMA decisions were included in our analysis. Consensus regarding ATC was reached

for anatomical main group for each medicine that did not have a unique entry in the WHOCC database. For two medicines (Eritoran and Clazosentan), no therapeutic subgroup could be assigned.

Pediatric development was agreed for 358 medicines and waived for 173 medicines (Figure 1). Antibacterials for systemic use, antihemorrhagic agents, antineoplastic agents, drugs used in diabetes, immunosuppressants, systemic antivirals, and vaccines accounted for half of all medicines for which pediatric development was agreed. In all drug classes, fewer medicines were researched in infants and toddlers than in older age groups, with the exception of antiparasitic agents, which was an extremely small drug class in the analysis. Medicines were researched at the same frequency in children and adolescents for nearly all drug classes, with the exception of drugs that act on genitourinary system and sex hormones, which were studied more frequently in adolescents. Deferral of one of the measures described in the PIP was granted for 83% of medicines with agreed pediatric development.

The majority of medicines were formulated for parenteral (48%) or oral (43%) use (allergens not considered). Oral formulation types are shown in Figure 2. Multiple dosage formulations were listed for 124 medicines, covering a range of 2-7 dosage formulations. Age-appropriate formulations were considered for 210 medicines for infants and toddlers, for 327 medicines for young children 327, and for 331 medicines for adolescents.

Drug classes with an established or potential pediatric need

A total of 323 active substances in 51 different therapeutic subgroups had a recognized need for pediatric research or development; full pediatric development was indicated for half of these active substances (Figure 3). Drugs used in diabetes, drugs for cardiac therapy, antineoplastic agents, immunosuppressants, anesthetics, analgesics, antiepileptics, psycholeptics and drugs for obstructive airway disease accounted for half of all medicines for which a need for pediatric research or development was recognized.

Trends in the prevalence of drug use in Dutch children

From 2005 through 2011, the prevalence of using any medicine was highest among adolescents, infants, and toddlers. Whereas the prevalence increased in adolescents, the use of medicines in infants and toddlers decreased after 2008. Use in children remained relatively stable in any calendar year after an initial increase from 2005 to 2006. The prevalence of medication use per age group is shown in Figure 4.

Trends in pharmaceutical expenditure data

Pharmaceutical expenditure for all drug classes was generally stable in the Netherlands from 2007 through 2011. Notable exceptions included a clear decrease in the cost of cardiovascular drugs, a clear increase in the cost of antineoplastic and immunomodulating drugs, and varying costs for neurological agents and drugs that act on the alimentary tract. The per-user and the total (volume times per-user) costs are shown in Figure 5.

Survey of Dutch physicians working with children

The majority of respondents (19 out of 32 who provided basic information) worked (at least part-time) at a non-academic center. The survey related to ophthalmology was the only one not completed once. Incomplete surveys included surveys with a large number of medicines, for example the survey related to cardiovascular disease. Surveys completed by at least 10% of respondents included those related to neonatology/pediatric intensive care, cardiovascular diseases, diagnostics, anesthesiology, and pain. These surveys included a total of 36 different active substances, five of which (chloroprocaine, perflubutane, fentanyl citrate, morphine, and dopamine) were perceived as useful by all respondents. In most cases, if the respondents identified the medicine as being useful, they also found research to be useful and would use the

medicine in clinical practice. Another 13 active substances were perceived as not useful by all of the respondents for the proposed indication, nearly all of which were listed for treating cardiovascular disease.

Discussion

More than five years have passed since the Pediatric Regulation was implemented; in this period, the EU budget's contribution to operational costs totaled more than 39 million euros, in addition to the contribution of resources in-kind by European national competent authorities[18]. Despite this substantial public health investment, it is unlikely that (future) pediatric authorizations will be focused more on pediatric needs. Drug classes and therapeutic subgroups with a high need for pediatric research and development on the EMA Needs Lists (including drugs that act on the cardiovascular or nervous system and drugs for treating obstructive airway disease) or for which a pressing pediatric need has been expressed in the literature (e.g., ophthalmological agents[19]) are either researched rarely or often waived from pediatric development. In addition, despite the diversity of therapeutic subclasses listed on the Needs Lists, pediatric research agreed under the Regulation was dominated by seven therapeutic subgroups (allergens not considered).

Ideally, drug research in children should be prioritized based on current trends in medication use[20] and frequent pediatric use of medicines that are not readily available or age-appropriate may indicate an important unmet pediatric need[1]. Our analysis of Dutch outpatient pediatric use revealed that the use of any medicine was highest among Dutch adolescents, infants, and toddlers, which suggests a shift in the highest use of medication towards adolescents, as previous studies found the highest prevalence of medication use among younger children[16,21]. Consistent with this notion, adolescents were rarely waived from pediatric development under the Regulation. In 2009, the percentage of medicines that were authorized and commercially available for use in Dutch children was highest for anti-infectives, respiratory drugs, and antiparasitic agents, and lowest for genitourinary drugs, sex hormones, dermatologicals, and cardiovascular drugs[1]. Although future studies should attempt to determine whether the low availability of these drug classes for children is a problem in clinical practice[1], combining these availability data with our outpatient usage data suggests the possible presence of an unmet pediatric need for research and development of dermatological agents in all ages and genitourinary drugs and sex hormones in adolescents; although these medicines are frequently prescribed to these age groups, they are not always readily available. Unfortunately, these drug classes are researched relatively rarely under the Regulation. Based on prescription and availability data, there is no apparent pediatric need for anti-infectives (which are prescribed frequently and are readily available), a drug class that is researched frequently under the Regulation. However, current availability data may not be the best indicator of the potential need for anti-infectives, as the need for drug availability may exceed regulations in the near future due to microbial (antibiotic resistance) and viral (pandemic flu) threats. Therefore, the development of new anti-infectives for use in children should not lag behind the development for use in adults.

The need to develop more age-appropriate formulations for younger age groups[1] appears to be addressed by the Regulation, as infants and toddlers are considered with respect to more than 200 medicines. In contrast to expectations[6], in most cases only one formulation type will be developed. Despite the inclusion of younger age groups, there seems to be no shift in the types of formulations that may be available for children following the implementation of the Regulation; similar to the situation in 2009[1], most pediatric medicines that are developed are intended for oral (mostly tablets) or parenteral administration.

The Regulation may lead to newer, safer, or more targeted treatments, as our study database included several novel drug types which may also become available for use in children, including tissue transglutaminase inhibitors[22], soluble guanylate cyclase activators[23], and toll-like receptor 4 receptor antagonists[24]. In addition, several protein-based drugs have agreed pediatric

development plans and may provide additional options for meeting unmet pediatric needs[25]. It remains to be seen whether these drugs will be developed successfully for children, as some developments have already been discontinued[10]. In addition, as more than 80% of the medicines in our database had at least one deferred measure in the agreed development plans, children are likely to have late rather than early access to new medicines. As a result, it is possible that no effect of the Regulation will be seen regarding the total off-label use in the pediatric population – even though this is one of the Regulation's intended aims – as has been demonstrated for triptans, which were labeled for pediatric use with delay; pediatric treatment remained dominated by off-label use, despite labeling the product in an age-appropriate formulation for the most relevant age group[26].

Finally, relatively few medicines in the fields of neonatology/pediatric intensive care, cardiovascular disease, diagnostics, pain, and anesthesiology were considered to be needed by a small contingent of Dutch physicians working in pediatric healthcare.

It is not surprising that the drug classes in our evaluation with the lowest number of medicines with agreed pediatric development have the lowest pharmaceutical expenditure. Under the Regulation, only medicines for which the adult indication also exists in children and for which no important reason exists to waive pediatric development will be considered for pediatric development. It has been argued that many children are denied access to innovative medicines because of this 'adult-driven' approach, for example in the field of oncology[27]. It has therefore been argued that implementation of the Regulation should be guided instead by the biology of pediatric tumors and the medicine's mechanism of action. We argue that this approach should be expanded beyond pediatric oncology, as this approach is relevant to other therapeutic areas as well. Indeed, in a recent publication, the PDCO explained that it tends to ask for research based on the medicine's mechanism of action[28]. However, whether this request for additional research will be voluntary or mandatory is currently unclear. In addition, new incentives should be considered for first-in-children indications.

It should be noted that our evaluation was limited by the cross-sectional nature of the collected data, as agreed PIPS may have been modified after our search. In addition, some data in the EMA database cannot be readily used for scientific analyses such as our study. For example, although duplicate applications and shared pediatric development among PIPS cannot be identified easily, this information is important for reliably assessing the impact of the Regulation on pediatric research and development. Our evaluation of the perceived usefulness of medicines researched under the Regulation among Dutch physicians working in pediatric healthcare should be regarded as preliminary, as it included fifty physicians only. In addition, it may be valuable to evaluate the perceived usefulness in expert groups as well.

In conclusion, progress has been made regarding the (planned) inclusion of adolescents in pediatric research and the development of more age-appropriate formulations for younger age groups. However, the Regulation's key strategy does not necessarily lead to the increased pediatric development of drug classes for which there may be a unmet pediatric need and physicians are unlikely to have more options in terms of formulation types for the majority of pediatric medicines. Instead, the Regulation's output is in line with expenditure data, most likely as a result of the 'adult-driven' approach. Pediatric research in actual needs areas may be hampered as a result of the Regulation, as the number of agreed pediatric development plans is high and the number of children available for research is low. Given the substantial public health investment and the potential negative effects of the Regulation on research in actual needs areas, important refinements in implementation are needed in order to ensure that the Regulation will provide children with the medicines they actually need.

REFERENCES

1 van Riet-Nales DA, de Jager KE, Schobben AF, Egberts TC, Rademaker CM. The availability and age-appropriateness of medicines authorized for children in The Netherlands. *Br J Clin Pharmacol.* 2011;72:465-473.
2 Pandolfini C, Bonati M. European paediatric research and children's therapeutic needs. A trial review. *Acta Paediatr.* 2008;97:1232-1237.
3 Pandolfini C, Bonati M, Rossi V, Santoro E, Choonara I, Naylor C, et al. The DEC-net European register of paediatric drug therapy trials: contents and context. *Eur J Clin Pharmacol.* 2008;64:611-617.
4 Rocchi F, Paolucci P, Ceci A, Rossi P. The European paediatric legislation: benefits and perspectives. *Ital J Pediatr.* 2010;36:56.
5 Paolucci P, Jones KP, del Carmen Cano GM, Catapano M, Iolascon A, Ceci A. Challenges in prescribing drugs for children with cancer. *Lancet Oncol.* 2008;9:176-183.
6 Breitkreutz J. European perspectives on pediatric formulations. *Clin Ther.* 2008;30:2146-2154.
7 European Medicines Agency with its Paediatric Committee. 5-year Report to the European Commission. General report on the experience acquired as a result of the application of the Paediatric Regulation. EMA/428172/2012, 1-187. 7-8-0012.
8 European Medicines Agency. Database including Opinions and Decisions on Paediatric Investigation Plans. 2013.
9 WHOCC database, online. 2014. 2014.
10 Integrity Database. 2014.
11 WHOCC list of new ATC codes. 2014.
12 European Medicines Agency (PDCO). Pediatric Needs Lists. 2013.
13 Herings RMC, Pederson L. Pharmacy-based Medical Record Linkage Systems. In: Strom BL, Kimmel SE, Hennessy S, editors. Pharmacoepidemiology. 5th Edition ed. John Wiley & Sons, Ltd.; 2012. 270-286.
14 van Herk-Sukel MP, van de Poll-Franse LV, Lemmens VE, Vreugdenhil G, Pruijt JF, Coebergh JW, et al. New opportunities for drug outcomes research in cancer patients: the linkage of the Eindhoven Cancer Registry and the PHARMO Record Linkage System. *Eur J Cancer.* 2010;46:395-404.
15 PHARMO. Internal PHARMO documents. 2012.
16 Schirm E, van den Berg P, Gebben H, Sauer P, De Jong-van den Berg. Drug use of children in the community assessed through pharmacy dispensing data. *Br J Clin Pharmacol.* 2000;50:473-478.
17 GIPdatabank. 2012.
18 European Commission. Better Medicines for Children: From Concept to Reality. Progress Report on the Paediatric Regulation (EC) No 1901/2006. 2013 Jun 24.
19 Fortinguerra F, Clavenna A, Bonati M. Ocular medicines in children: the regulatory situation related to clinical research. *BMC Pediatr.* 2012;12:8.
20 Boots I, Sukhai RN, Klein RH, Holl RA, Wit JM, Cohen AF, et al. Stimulation programs for pediatric drug research--do children really benefit? *Eur J Pediatr.* 2007;166:849-855.
21 Sturkenboom MC, Verhamme KM, Nicolosi A, Murray ML, Neubert A, Caudri D, et al. Drug use in children: cohort study in three European countries. BMJ. 2008;337:a2245.
22 Badarau E, Collighan RJ, Griffin M. Recent advances in the development of tissue transglutaminase (TG2) inhibitors. *Amino Acids.* 2013;44:119-127.
23 Stasch JP, Pacher P, Evgenov OV. Soluble guanylate cyclase as an emerging therapeutic target in cardiopulmonary disease. *Circulation.* 2011;123:2263-2273.
24 Leon CG, Tory R, Jia J, Sivak O, Wasan KM. Discovery and development of toll-like receptor 4 (TLR4) antagonists: a new paradigm for treating sepsis and other diseases. *Pharm Res.* 2008;25:1751-1761.
25 Xu Z, Davis HM, Zhou H. Rational development and utilization of antibody-based therapeutic proteins in pediatrics. *Pharmacol Ther.* 2013;137:225-247.
26 Lindkvist J, Airaksinen M, Kaukonen AM, Klaukka T, Hoppu K. Evolution of paediatric off-label use after new significant medicines become available for adults: a study on triptans in Finnish children 1994-2007. *Br J Clin Pharmacol.* 2011;71:929-935.
27 Vassal G, Geoerger B, Morland B. Is the European pediatric medicine regulation working for children and adolescents with cancer? *Clin Cancer Res.* 2013;19:1315-1325.
28 van den Berg H, Torqui A, Wesseling AI. [Five years of European regulation on medication use in children: what has been achieved?]. *Ned Tijdschr Geneeskd.* 2013;157:A6000

FIGURE 1 Number of active substances per drug class for which pediatric research and development was agreed (with or without deferral of one of the measures in at least one development plan) or waived under the Pediatric Regulation, based on published EMA decisions through March 2012. Octocog alfa (B02), cyclosporin (S01), budesonide (R03), ulipristal acetate (G03), everolimus (L01, L04), and afamelanotide (D02) had agreed as well as waived pediatric development plans. Antineoplastic agents, immunosuppressants, antihemorrhagic drugs, antiviral agents for systemic use, and vaccines were the largest therapeutic subgroups for which pediatric development was agreed (each subgroup contained >20 active substances). In the main drug classes for which pediatric development was waived, drugs that act on the renin-angiotensin system, lipid-modifying drugs, antineoplastic drugs, drugs used in diabetes, sex hormones and modulators of the genital system, and ophthalmological drugs were the largest therapeutic subgroups (each subgroup contained >10 active substances).

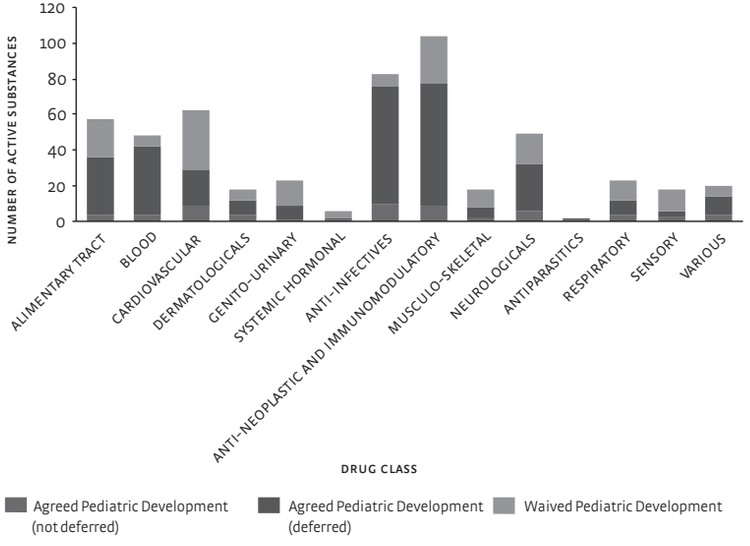


FIGURE 2 Oral formulation types developed or researched under the Pediatric Regulation, based on published EMA decisions through March 2012.

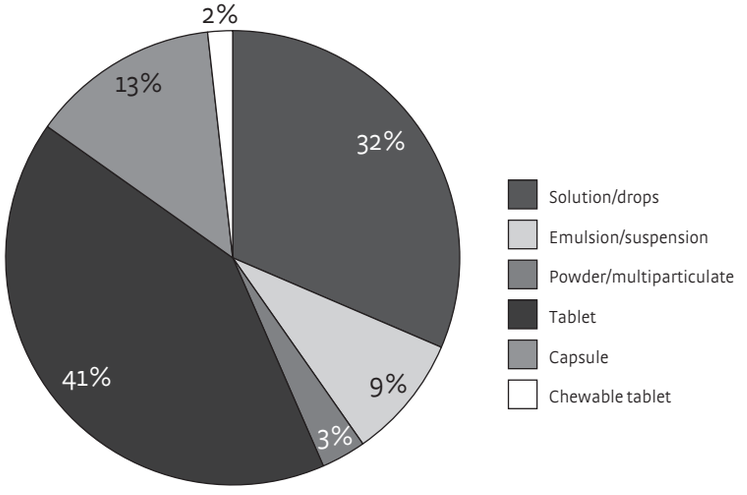


FIGURE 3 Number of active substances per drug class listed in the Pediatric Needs Lists published through July 2012 for which a pediatric need for research was indicated. For three neurological agents, no specific need was specified on the Neurology Needs List. Drugs that act on the sensory organs were not included in any of the Pediatric Needs Lists. Drugs that act on the sensory organs were not included in any of the Pediatric Needs Lists. Most of the active substances with a need for pediatric research or development were drugs used in patients with diabetes, antithrombic agents, cardiac therapy drugs, diuretics, anti-viral agents for systemic use, antineoplastic agents, immunosuppressants, anesthetics, analgesics, anti-epileptics, psycholeptic agents, and drugs for treating obstructive airway disease (each therapeutic subgroup contained 10-38 active substances).

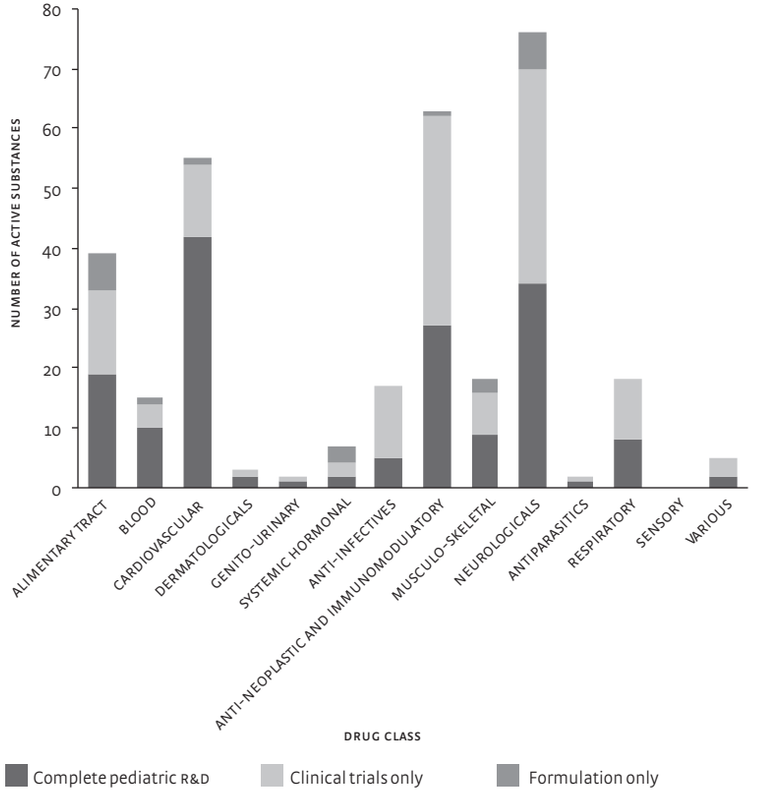


FIGURE 4 Average number of users per 10,000 children by anatomical class and age group from 2005 through 2011. In all pediatric age groups, the most frequently used medications were drugs that act on the alimentary tract, dermatologicals, anti-infectives for systemic use, and respiratory drugs. With respect to infants and toddlers, drugs that act on the sensory organs were also used frequently; among adolescents, drugs that act on the genitourinary system and sex hormones were used frequently (predominantly among females). In all pediatric age groups, a decrease in prevalence was observed with respect to the use of anti-infectives, and an increase in prevalence was observed with respect to drugs that act the alimentary tract and dermatological agents. Among children and adolescents, an increase in the use of neurological agents was observed, with the largest increase among antidepressants (including antidepressants combined with psycholeptics) and stimulants. Among adolescents, an increase in the use of respiratory drugs and drugs that act on the genitourinary system was also observed.

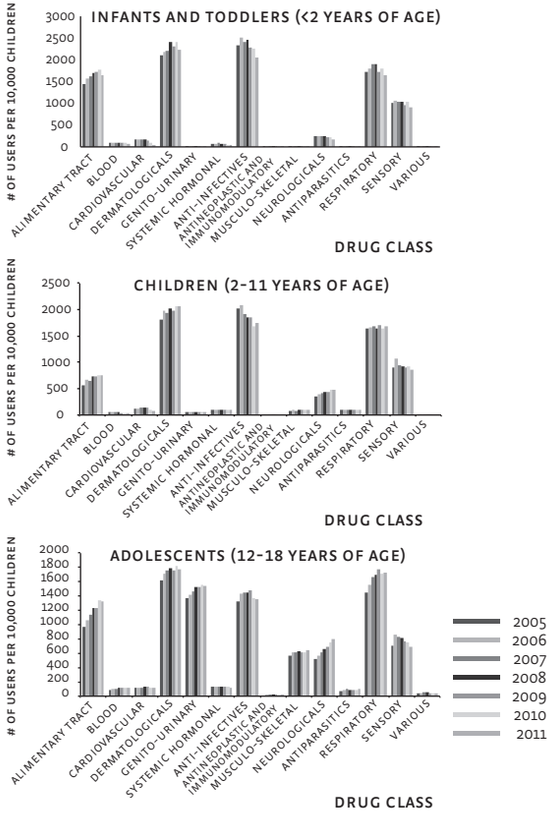
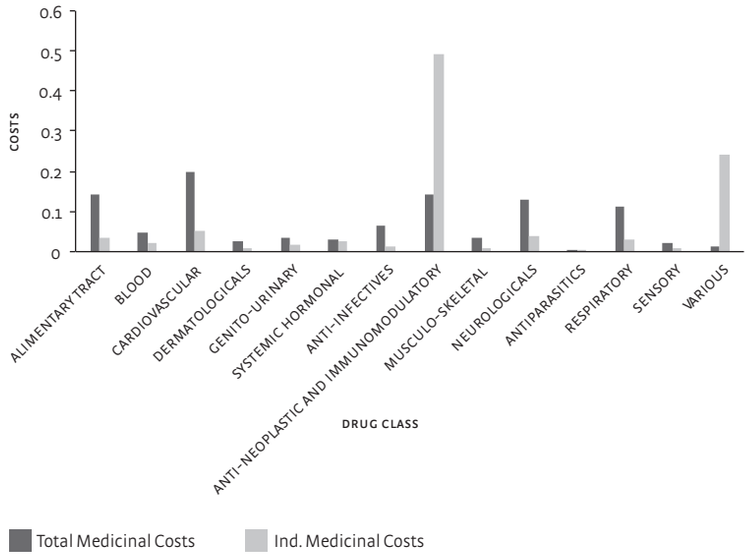


FIGURE 5 Total pharmaceutical expenditure data of active substances per drug class from 2007 through 2011; data were retrieved from the GIP databank and are expressed as the fraction of total costs of all drug classes for overall medicinal costs (total medicinal costs) and per user (individual medicinal costs).



CHAPTER 3

Biomarkers of acute methylphenidate effects in children and adolescents with attention-deficit/ hyperactivity disorder

Submitted

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ABSTRACT

Despite the extensive use of methylphenidate (MPH) and considerable research, suitable validated biomarkers for monitoring the effects of MPH are not currently available. Here, we performed a systematic literature review to identify generally applicable biomarkers for monitoring the effects of immediate-release MPH (MPH-IR) in children and adolescents with attention-deficit/hyperactivity disorder (ADHD). We identified 78 randomized placebo-controlled clinical studies that investigated central nervous system effects following a single dose of MPH-IR in pediatric ADHD patients. Neurocognitive clusters and individual tests that were used in five or more studies were evaluated for reporting consistent MPH effects. The following outcomes showed a consistent response to a therapeutic MPH dose across studies based on different cohorts: Continuous Performance Test, Go/no-go Task, Visual Evoked Potentials, and several observation scales (including Following Rules Observations, Oppositional Behavior Observations, On-Task Behavior Observations, and Impulsivity Behavior Observations). A closer inspection of the Visual Evoked Potentials revealed that MPH mediates increases in late potential amplitudes. MPH's effect was best detected in tests and observations regarding motor control, sustained attention, divided attention, and impulsivity (inhibitory control), indicating that MPH has acute effects on all three core symptoms of ADHD among MPH-responsive children with ADHD. These candidate biomarkers should be investigated further in future studies to obtain a more thorough evaluation of dose-response relationships, including their effect size and potential applicability for predicting the response to MPH.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is the most common neurobehavioral disorder among children and adolescents. ADHD is characterized by the childhood onset of symptoms that include inattention and impulsivity/hyperactivity[1,2]. The psychostimulant methylphenidate (MPH) is the most commonly prescribed medication for treating the symptoms associated with pediatric ADHD[3,4]. This treatment has been validated by numerous controlled studies that show the efficacy of low-dose oral MPH at reducing the behavioral symptoms associated with the disorder; the effects are usually reported by the child's parents and teachers and include both the cognitive (inattention and impulsivity) and non-cognitive (hyperactivity) domains. In controlled clinical trials, approximately 60-70% of treated children show clinical improvement[5], although the response rate is lower (approximately 50%) and less predictable in clinical practice. The clinical use of MPH is usually based on a trial-and-error approach before optimal therapy is achieved; this approach is often used because MPH's effects vary widely between individuals in terms of clinical response[6] and pharmacokinetics (PK). More than 30% of patients do not respond favorably to MPH at any dose[6] and therefore must switch to an alternative medication after the initial titration phase. As a consequence of this lack of response, a significant percentage of ADHD children either experience a considerable delay in receiving adequate treatment or stop seeking treatment altogether. Therefore, there is a need for a more sensible, personalized therapeutic approach to ADHD.

Despite its long history of use, MPH's precise mechanism of action remains poorly understood, complicating the assessment of treatment efficacy or the timely identification of non-responders. MPH is available in several formulations, with a variety of delivery mechanisms that result in changing PK and effect profiles throughout the day[7]. Immediate-release MPH (MPH-IR) has been on the market for more than 50 years, whereas other formulations have only recently become available. MPH-IR is a short-acting compound, with an onset of action within 30-60 minutes and reaching peak clinical effect 1-2 hours after administration; the effects typically last 2-5 hours. The relationship between MPH's PK and biological effects is complex and not completely understood; however, recent studies suggest that the drug's principal clinical effects closely follow its predicted PK profiles[8,9]. The autonomic, psychomotor, and neurocognitive effects of MPH-IR in pediatric ADHD have been studied extensively. However, previous published studies of the effects of MPH-IR have yielded conflicting results due to several sources of variability, including a lack of standardized biomarkers and effect measures for MPH[10,11]. Despite the widespread use of MPH and extensive research, no validated or generally accepted biomarkers for MPH's effects in children and adolescents with ADHD have been identified. Identifying suitable biomarkers will help researchers develop a more efficacious and specific treatment regimen for children with ADHD; for example, biomarkers could be used for the early identification of responders versus non-responders, to identify patients with an increased risk of developing adverse side effects, and to monitor treatment outcome. Biomarkers could also facilitate future research regarding the core pathology of ADHD, MPH's mechanism of action, and the effects of stimulants in children. The following operational criteria have been used in the search for a suitable biomarker[12–16]: 1: a clear, consistent response across studies using different study cohorts; 2: a clear change in the biomarker in response to a therapeutic dose of MPH; 3: a measurable dose-response and/or concentration-response relationship; and 4) a plausible relationship between the biomarker, the pharmacology of MPH, and the pathogenesis of ADHD.

Here, we performed a systematic review of the literature to investigate generally applicable outcome measures in response to MPH-IR treatment of children and adolescents with ADHD. Our research group and other groups have successfully used this approach in healthy volunteers to identify suitable functional biomarkers for antipsychotic drugs[12], benzodiazepines[13], selective serotonin reuptake inhibitors[14], tetrahydrocannabinol[15], MDMA (i.e., ecstasy)[16], and alcohol[17].

Methods

Search strategy and data collection

To identify studies that investigated the acute effects of MPH in pediatric patients with ADHD, the databases PubMed, EMBASE, and PsycINFO were searched (for the search queries, see Table 1). All returned citations were imported in a Reference Manager database, and duplicate records were removed from this database. Potentially relevant studies were selected by viewing the titles and abstracts. Non-relevant reports were discarded, and the full-length articles of all relevant or potentially relevant reports were obtained. The following inclusion criteria were then applied to this pool of potentially eligible studies: studies that were randomized, double-blind, placebo-controlled clinical trials; studies that compared the effects of an acute dose of MPH-IR versus placebo in previously MPH-treated or MPH-naïve children and/or adolescents with ADHD; studies with a minimum washout period of five half-lives; studies with a minimum cohort size of ten subjects per treatment arm; and studies that were published in English. To minimize population heterogeneity, studies that included subjects with mental retardation (IQ <70) and studies in which ADHD patients were selected for a specific comorbidity were excluded.

Data were extracted from the studies to a pre-established database template (Microsoft Excel). The extracted data included information regarding the study details (e.g., study design, number of subjects, wash-out period), subject characteristics (e.g., gender, age, ADHD type, comorbidity, previous exposure to MPH), medication given (e.g., formulation, dose), and outcome measures (e.g., the type of test, time interval between drug administration and the test, overall effect). Because effect measures and MPH doses varied among the studies, each effect measure at a certain MPH dose was considered an independent effect measure. Thus, the total number of effect measures evaluated was the combined sum of the number of studies, tests, and MPH doses.

Data analysis

Outcome measures were clustered to groups of related tests or test variants (referred to as 'clusters') in order to generate a reasonable degree of standardization across studies and tests. For example, the Arrow/Sound (In) Compatibility Task, Dichotic Listening Task, Divided Attention Task, and Dual Task were clustered under 'Divided Attention', which is part of the functional domain 'Attention'. This approach enabled us to preserve individual study data in the early stages of our analysis, thus enabling us to evaluate individual frequently used tests using uniform outcome measures. We then performed a progressive condensation of the results into logical clusters, thus providing a more general assessment of the drug's effects on groups of comparable tests or functional domains. This approach enabled us to evaluate the practical suitability of using a test as a biomarker in small-to-medium size studies. No effort was made to further quantify the level of statistical significance. Neurocognitive and neurophysiological tasks were categorized into clusters of related tests or test variants[12] using the compendiums of Strauss[18] and Lezak[19].

The clusters were further divided into six domains (referred to as 'domains') in accordance with the Strauss compendium[18], including executive functions, memory, attention, motor function, language, and perception. Physiological measurements (e.g., heart rate, blood pressure, pupil size, and electrodermal activity) were not included in this review. Event-related potentials (ERPs) and neuroendocrine measurements (e.g., cortisol, growth hormone, and prolactin) were also grouped into clusters and domains.

With respect to ERPs, the following clustering method was used. A potential is evoked using a standard visual or auditory stimulus and an infrequent deviant stimulus (the 'target' in the Continuous Performance Task, Go/no-go task, and Oddball task). If the pattern of the potential evoked by the standard stimulus was significantly altered following MPH treatment, the event was classified as a significant effect of MPH on the neurophysiologic parameter (drug condition

x standard stimulus), regardless of the direction of the effect (i.e., a positive or negative wave, increased or decreased amplitude, or a change in time). In addition, if the pattern of the potential evoked by the deviant (or target) stimulus was altered significantly after MPH and compared to the standard stimulus (drug condition x standard stimulus x deviant stimulus), the response was considered to reflect an effect of MPH in the task-related neurocognitive domain (for example, sustained attention in the event of an ERP with the Continuous Performance Task) and was clustered as such in the database.

In several studies, subjective measures and rating scales were used to assess the effect of MPH in children with ADHD. Clustering of these measures was guided by a review of classification methods in published rating scales (the Conners Rating Scale[20] and scales derived therein, and the ADD-H Comprehensive Teacher Rating Scale[21]) and the clusters and domains that are used to classify the neurocognitive tasks[18,19]. This subdivision is described in the Results section.

In our analysis, we assumed that in most cases, no single consistent quantitative outcome parameter could be recorded for an individual test due to the large variation in methods and parameters. Therefore, the ability of a test to reveal a statistically significant difference between placebo and baseline was scored as '+' (an improvement or increase), '=' (no significant effect), or '–' (an impairment or decrease). Different parameters of a single test were grouped if they provided information regarding a common functional cluster. Several tasks yielded different outcome parameters, in some cases showing opposite effects of MPH. If these opposite responses were part of the same neurocognitive cluster, two items were scored for the same test (e.g., one '+' and one '-'). In cases in which one of the improved parameters was part of a different functional cluster than the parameter that did not improve, both items were scored separately (i.e., as belonging to separate clusters). Items that were considered to be secondary test parameters were marked with an asterisk in the database. For example, in the Continuous Performance Task, the level of commission errors (which evaluates impulsivity) is secondary to the level of omission errors (which evaluates sustained attention). These secondary parameters were clustered separately in secondary clusters. However, if a secondary parameter could be categorized in the same functional domain as the primary test parameter, it was not included separately, and only the primary item was added to the database.

For each outcome measure and functional cluster, we calculated the percentage of improved (+) or impaired (-) test outcomes relative to the total number of tests. Secondary outcome measures/clusters and ERP-related clusters were evaluated separately. Consistent MPH-induced improvement in task performance, rating, or observation was defined as an increase in >60% of the tests.

Dose-effect relationships

On average, the clinical dosing range for MPH is linearly correlated with the reduction in ADHD symptoms[22]. The likelihood that a given test will detect a difference in effect between MPH treatment and placebo is expected to increase with increasing dose. Therefore, to investigate MPH's dose-effect relationship, the doses were categorized as 'low', 'medium', or 'high'. The 'low' dose was defined as 0.1-0.25 mg/kg (i.e., the recommended daily therapeutic starting dose), the 'medium' dose was defined as 0.25–0.59 mg/kg, and the 'high' MPH dose was defined as ≥0.6 mg/kg[23]. Dose-effect relationships were measured for clusters and for effect measures that were used in ≥3 studies (per dose category) and/or across ≥15 studies. Dose-related changes in the average percentages of improvement were reported without formal statistical analyses.

Results

Identified studies

The literature search included all scientific articles that were published through December 31, 2009. The search yielded a total of 1,973 hits, from which

78 articles were identified that met the inclusion criteria for this review. Ten of these articles did not explicitly mention DSM or ICD criteria when describing the study population; however, these studies did mention the use of other diagnostic methods (for example, questionnaires, interviews with parents, etc.) to confirm the presence of a hyperactivity disorder. The majority of studies included boys ranging from 5-13 years of age, and the majority of studies investigated a 'medium' dose of MPH (defined as 0.25-0.59 mg/kg; see Methods). The study characteristics are summarized in Table 2.

Clustering of outcome measures

The identified outcome measures – categorized by functional cluster and domain – are summarized in Table 3. Results from domains and clusters that were used only once or twice could not be generalized, and will not be discussed further. In total, 151 separate outcome measures (i.e., tasks and observations) were used to assess the effect of MPH; 104 of these measures were used only once. Only 11 measures were used more than five times, with Visual Evoked Potential and the Go/no-go Task being the most frequently used measures (each was used in ten studies). Progressive condensation of outcome measures resulted in 49 clusters.

Following MPH treatment, task performance and observations generally improved. Only four of the 78 studies reported performance impairment or decreased hormone serum levels following MPH; these four outcomes were the Math Cheating Task[24], the Behavior After Failure Task[25], the Tachistoscopic Task[26], and Prolactin levels[27].

The outcome measures that were used in at least five studies (Table 4) were reasonably consistent (>60%) at detecting MPH's effects across studies (with the exception of the Arithmetic Task and the Reaction Time Task). The tests with the highest consistency were Visual Evoked Potentials and the Continuous Performance Task (with 84.2% and 76.2% consistency, respectively). With respect to the observational outcome measures, the following observation scales had the highest consistency, ranging from 68.4-100%: Following Rules, Oppositional Behavior, On-Task Behavior, and Impulsive Behavior. The only scale that did not improve consistently across the studies was Social Behavior. None of the outcome measures that were used in at least 5 studies showed impairment following MPH treatment.

Table 5 reports on the functional clusters (following categorization of outcome measures; see Methods) that contain all of the most consistent individual outcome measures identified above. Nearly half of the functional clusters that were assessed showed consistent improvement (i.e., >60% consistency) following MPH compared to placebo; these clusters include Divided Attention, Secondary Reaction Time, Sustained Attention, Motor Control, Evoked Potentials, and the following scales: Attention, Oppositional, ADHD, and Impulsivity. The highest consistency was seen for Evoked Potentials, Sustained Attention (Vigilance), and the Oppositional and Attention Scales. The observation-related outcome measures and functional clusters ('scales') were more sensitive at detecting the effects of MPH than task-related measures and clusters. Moreover, executive functioning-related clusters had relatively low improvement ratings.

With respect to evoked potentials, MPH-related changes in outcome measures and/or clusters were evaluated regardless of the direction of change (e.g., positive or negative, increased or decreased). Visual evoked potentials were measured in ten studies[28-37], and late potential amplitudes (≥300 msec) increased in approximately 70% of cases following MPH treatment. In contrast, we found no consistent change in early potentials or evoked potential latencies following MPH treatment. Auditory evoked potentials were measured in three studies[29,30,38] and showed a similar change following MPH treatment.

Dose-response relationships

We next investigated the dose-response relationship of the outcome measures and functional clusters that had a consistent MPH-induced response (>60% consistency). Specifically, we examined the dose-response curve of each outcome that was used in ≥3 studies per dose level (low, medium, or high;

see Methods) or ≥15 studies (Table 6). The outcome measures Go/no-go Task and Scale – ADHD showed reasonably high consistency for all dose levels. The functional cluster Sustained Attention (Vigilance) had a robust dose-response relationship in the medium and high dose levels. The cluster Motor Control also showed an increasing response in the high dose level; in contrast, the low and medium doses gave similar response rates across the studies.

Discussion

The clinical use of methylphenidate (MPH) for treating children with ADHD is currently based on a trial-and-error approach. The availability of MPH efficacy biomarkers may help clinicians determine the optimal type and dose of medication, thus providing the most efficacious treatment regimen. Here, we performed a systematic literature review in order to identify potential biomarkers for assessing the acute effects of a single dose of immediate-release MPH in children and adolescents with ADHD. Using this approach, we expected to identify the most sensitive outcome measures for the effect of MPH, provided that a change in task performance, rating, or observation can be detected after administering a single low dose of MPH; moreover, the responses that were consistently detected in multiple studies should reflect the most robust biomarkers. Outcome measures were clustered into groups of related tests and/or test variants in an attempt to standardize the results across studies and tests, and this was followed by a progressive condensation of results into 20 clusters of related CNS tests. Although 151 different outcome measures were used to assess the effects of MPH, fewer than one-third (47 measures) were used in more than one study. The Continuous Performance Test, Go/no-go Task, and Visual Evoked Potentials, as well as several observation scales – including Following Rules Observations, Oppositional Behavior Observations, On-Task Behavior Observations, and Impulsivity Behavior Observations – were identified as outcome measures that showed a clear, consistent response to therapeutic MPH doses in several studies performed by various research groups. We also observed consistent MPH-induced improvement at the cluster level for Divided Attention, Sustained Attention (Vigilance), Reaction Time, Motor Control, Evoked Potentials, and several scales (Attention, Oppositional, ADHD, and Impulsivity).

In order to be useful in a clinical setting, a biomarker should be sufficiently sensitive to detect the effect of a therapeutic dose of MPH; moreover, a plausible relationship between the biomarker, MPH pharmacology, and/or ADHD pathogenesis should also exist. Several neurotransmitters have been implicated in the pathophysiology of ADHD (and by extension, MPH's mechanism of action). Compelling evidence suggests that dysfunctional dopamine and norepinephrine neurotransmission, as well as dysregulation of dopaminergic pathways, are involved in the pathogenesis of ADHD[39,40]. MPH appears to stimulate the dopaminergic and noradrenergic systems in the fronto-striatal region of the brain, thereby improving symptoms associated with impaired motor and cognitive function. Other neurotransmitters (such as histamine, and serotonin) and nicotine have also been suggested to play a role in the pathophysiology of ADHD[41-45], and both animal and human studies have reported increased levels of these neurotransmitters in the prefrontal cortex following MPH treatment. However, the evidence collected to date does not necessarily support the putative relationship between MPH-induced neurochemical modulation and the clinical improvements observed in ADHD patients following MPH treatment[39,46]. In our study, all of the MPH-sensitive outcome measures – and most of the functional clusters – were associated with the core symptoms of ADHD. Other functional clusters that were previously associated with ADHD pathogenesis and were measured in >5 studies (e.g., working memory, reasoning, and set shifting)[47] failed to show consistent MPH-induced improvements. This finding seems to correspond with the clinical finding that a single therapeutic dose of MPH selectively improves some of ADHD's core features (in particular, attention, impulsivity, and hyperactivity), whereas learning, planning, and organization improve only following chronic MPH treatment (possibly as an indirect consequence of MPH's acute CNS effects). ADHD has also been associated with various deficits in event-related potentials. In the auditory modality,

ADHD-related differences are apparent in all components from the auditory brain-stem response to the late slow wave. Although relatively few studies have investigated the visual attention system, similar differences have been reported for a range of components; for example, the visual P3 component has been reported to differ between children with ADHD and control subjects[48]. Late evoked potentials are associated with stimulus evaluation and matching procedures that are related to attention[37,49]. Our findings show that MPH consistently induces a change in late evoked potential amplitudes.

Previously reported MPH-induced improvements include improvements in impulse control, learning, short-term memory, and activity[10], with the largest improvements in activity level, attention, and inhibition[50]. Pietrzak and colleagues[11] used an approach similar to ours, but their search was limited to cognitive tasks, and they included both non-controlled trials and chronic MPH trials, which may explain the differences between their results and our results. For example, Pietzrak and colleagues reported that saccadic eye movement, planning/cognitive flexibility, attention/vigilance, and inhibitory control were improved by MPH in ≥70% of the studies they reviewed. In contrast, only 50% of the studies they reviewed reported improvements in working memory and divided attention following MPH treatment. In our study, tasks and/or observations that evaluate learning and planning could not be reviewed in our study, as they were not used frequently enough in the studies we reviewed. A recent systematic review and meta-analysis of the effects of MPH on cognitive function[51] found that MPH improves executive and non-executive memory, reaction time, reaction time variability, and response inhibition in children and adolescents with ADHD. In contrast to our study, they included studies with additional non-pharmacological interventions (as long as these interventions were applied to both placebo-controlled randomized study groups); in addition, for studies that included several doses of MPH, they included only the data regarding the highest MPH dose, which may explain their positive findings with respect to memory. Other potentially sensitive biomarkers for MPH effect not included in our database include cognitive performance (sustained attention/vigilance)[52], baseline autonomic arousal (heart rate and blood pressure)[52], and baseline brain activity (EEG theta power)[52], and the contingent negative variation (CNV, a slow negative shift in the EEG that can occurs between a warning signal and an imperative stimulus during a reaction time task)[53].

Ideally, a suitable research biomarker should have a clear dose-response relationship with MPH and should preferably be sensitive to a low therapeutic dose of MPH, criteria that were not addressed in previous reviews. Here, we predefined three discrete dose ranges. The majority of studies in our review used the medium dose range (0.25-0.59 mg/kg), which limited our ability to assess a putative dose-response relationship. The cut-off values for the dose categories were based on clinically relevant ranges. Alternatively, we could have determined the cut-off values based on the distribution of dose levels in our dataset, thereby balancing the number of studies in each dose category. However, this approach would have been *post hoc* (rather than *a priori*) and would not have reflected true clinical practices. In our data set, dose-response relationships were determined for the outcome measures and functional clusters that were sensitive to the effects of MPH in a minimum number of studies. Because our method merely determined the statistical significance of test results following treatment, it did not enable us to detect effect sizes; rather, we could only determine consistency in the measured outcomes across studies. This approach may have masked the identification of a dose-response relationship, particularly for biomarkers that showed a clear response at all dose levels, including the lowest level (for example, with outcome measures from the Go/no-go Task, the functional cluster Motor Control, and outcome measures of the scale ADHD).

Our analysis has several limitations. Firstly, the basic concept of ADHD as a disorder changed several times in terms of both nomenclature and classification in the past few decades. As a result, potentially eligible studies may have been missed. In addition, although differences in the response to MPH have been described between the inattentive/hyperactive and combined subtypes of ADHD[54–56], we did not attempt to differentiate between ADHD subtypes, due in part to an insufficient number of studies. Also, publication bias might

have played a role[57], potentially increasing the publication success of studies that report significant MPH effects. Moreover, although grouping the tests into functional clusters and domains provided a degree of standardization, it also reduced the level of detail with respect to the information collected. Thus, potentially suitable biomarkers might have been missed, given that infrequently used outcome measures were excluded from further analysis.

Other limitations are inherent to the nature of the studies that have been published. Studies with a period of ≥5 half-lives between placebo and MPH occasions were included to ensure complete washout; however, in some studies, the effects of MPH on withdrawal – rather than MPH's effects on the condition itself – may have been measured. In addition, several studies used different criteria for selecting subjects, leading to potential differences in baseline impairment and subsequent differences in the magnitude of the response to treatment. Our search also returned studies in which subjects received prior treatment with MPH. Differences between ADHD patients who were previously treated with MPH and 'MPH-naïve' patients have been reported in networks associated with executive control[58] and dopaminergic metabolism[84], and this difference may have influenced our findings. In addition, several studies used a titration scheme; rather than controlling the MPH dose given before the study period, the children were stabilized with an optimal MPH dose (determined by the treating clinician) before evaluating the effect of the treatment. Patients receiving a high dose of MPH (i.e., 60 mg) have been reported to exhibit smaller changes in clinical measures during placebo treatment, suggesting that patients who require a higher dose of MPH are likely to have a worse outcome if left untreated. These subjects also exhibited higher sensitivity to drug treatment, reflected in smaller estimated EC_{50} values[59], consistent with previous reports that children with more behavior problems at baseline tend to respond better to MPH[9,60,61]. Moreover, the majority of studies included boys 5-13 years of age. Only a few studies (primarily the more recent studies) also included preschoolers and adolescents with ADHD and/or included a reasonable numbers of girls. A limited body of evidence suggests that aside from the preschool period[62,63], few age-related differences exist during childhood and adolescence with respect to the response and tolerance to MPH[64]. Because relatively few published studies specifically examined the moderating effect of gender, and because sample sizes were questionable in some studies, no clear conclusions can be drawn regarding whether gender affects the response to treatment[64,65]. Pietrzak and colleagues[11] reviewed several other factors of influence related to intra-individual and inter-individual differences in medication response, including the study design and repeated neuropsychological assessments. Our dose-response analysis was complicated further by several additional factors. For example, effect profiles can differ among outcome measures, including the effect's time of onset, duration, and time to reach maximal effect. The stimulant-induced reduction in motor activity can persist for up to 7-8 hours, whereas the drug's effects on attention last for only 2-3 hours[39,66]. Because most studies did not include repeated effect measurements, it is conceivable that in some studies the time points of specific measurement did not coincide with the time of maximal effect, thereby missing potentially significant improvements. In addition, previous studies reported evidence of acute tolerance to MPH[67,68]. MPH pharmacokinetics and/or pharmacodynamics also vary among children with ADHD. Thus, it is possible that some studies lacked a sufficiently large sample size to detect a significant effect. Although this might have been overcome by performing a formal quantitative meta-analysis, in nearly all cases this would have been hampered by the lack of uniform quantitative outcome parameters. The change from baseline following MPH treatment can also depend on the individual patient's baseline value (i.e., intrinsic state) and is estimated to account for approximately half of the variability observed in MPH's effects[69].

Nevertheless, despite these limitations, we identified several tests that could potentially serve as biomarkers for monitoring the acute effects of a single dose of MPH-IR in pediatric ADHD. The most reliable tests were related to ADHD's core features of motor control, attention and impulsivity, and event-related potentials. These potential biomarkers might help identify responders versus non-responders following a test dose of MPH. Because dose-effect relationships could not be quantified, these tests and clusters should be

investigated further in order to thoroughly evaluate the dose-response relationships, including effect size and, and establish clinically relevant changes. Our study revealed that these studies would benefit greatly from a certain degree of standardization. Ideally, these studies should include concentration- (in addition to dose-) effect relationships at several time points in order to profile the effect of MPH treatment in children and adolescents with ADHD. Short-term data is generally inadequate for assessing a long-term treatment response, as large-scale studies have shown that short-term response rates – which can be as high as 75-80% – often drop to 55-60% over the long run. Therefore, the predictive value of these candidate MPH biomarkers should be tested in long-term trials. In addition, performing multiple tests – rather than one test – may be preferred in such a long-term trial, as multiple tests can yield a more precise and stronger prediction of the response to MPH treatment and can account for the heterogeneity of ADHD[52]. This research should also include biomarkers for MPH tolerance, as many children who respond positively to MPH also experience adverse side events[70,71], potentially reducing treatment compliance or causing the patient to discontinue treatment altogether[70–72]. Finally, these putative MPH biomarkers could help guide future research regarding the core pathology of ADHD, MPH's mechanism of action, and the effects of stimulant use in children in general, as distinct classes of drugs can elicit distinct effect profiles[12–17].

REFERENCES

1 World Health Organization. ICD-10; International Statistical Classification of Diseases and Related Health Problems. 1999.
2 American Psychiatric Association. DSM-IV: Diagnosic and Statistical Manual of Mental Disorder. 4th ed. Washington, DC: The Association; 1994.
3 Greenhill LL, Pliszka S, Dulcan MK, Bernet W, Arnold V, Beitchman J, et al. Practice parameter for the use of stimulant medications in the treatment of children, adolescents, and adults. *J Am Acad Child Adolesc Psychiatry.* 2002;41:26S-49S.
4 Arnsten AF. Stimulants: Therapeutic actions in ADHD. *Neuropsychopharmacology.* 2006;31:2376-2383.
5 Greenhill LL, Pliszka S, Dulcan MK, Bernet W, Arnold V, Beitchman J, et al. Summary of the practice parameter for the use of stimulant medications in the treatment of children, adolescents, and adults. *J Am Acad Child Adolesc Psychiatry.* 2001;40:1352-1355.
6 Markowitz JS, Straughn AB, Patrick KS. Advances in the pharmacotherapy of attention-deficit-hyperactivity disorder: focus on methylphenidate formulations. *Pharmacotherapy.* 2003;23:1281-1299.
7 Swanson J, Volkow N. Pharmacokinetic and pharmacodynamic properties of methylphenidate in humans, in stimulant drugs and ADHD: Basic and clinical neuroscience. 259-82. 2001. New York: Oxford University Press 2001, Solanto MV, Arnsten AF, Castellanos FX, Eds.
8 Swanson JM, Wigal SB, Wigal T, Sonuga-Barke E, Greenhill LL, Biederman J, et al. A comparison of once-daily extended-release methylphenidate formulations in children with attention-deficit/hyperactivity disorder in the laboratory school (the Comacs Study). *Pediatrics.* 2004;113:e206-e216.
9 Sonuga-Barke EJ, Swanson JM, Coghill D, DeCory HH, Hatch SJ. Efficacy of two once-daily methylphenidate formulations compared across dose levels at different times of the day: preliminary indications from a secondary analysis of the COMACS study data. BMC *Psychiatry.* 2004;4:28.
10 Oettinger L, Jr., Majovski LV. Methylphenidate: a review. *South Med J.* 1976;69:161-163.
11 Pietrzak RH, Mollica CM, Maruff P, Snyder PJ. Cognitive effects of immediate-release methylphenidate in children with attention-deficit/hyperactivity disorder. *Neurosci Biobehav Rev.* 2006;30:1225-1245.
12 de Visser SJ, van der Post J, Pieters MS, Cohen AF, van Gerven JM. Biomarkers for the effects of antipsychotic drugs in healthy volunteers. *Br J Clin Pharmacol.* 2001;51:119-132.
13 de Visser SJ, van der Post JP, de Waal PP, Cornet F, Cohen AF, van Gerven JM. Biomarkers for the effects of benzodiazepines in healthy volunteers. *Br J Clin Pharmacol.* 2003;55:39-50.
14 Dumont GJ, de Visser SJ, Cohen AF, van Gerven JM. Biomarkers for the effects of selective serotonin reuptake inhibitors (SSRIS) in healthy subjects. *Br J Clin Pharmacol.* 2005;59:495-510.
15 Zuurman L, Ippel AE, Moin E, van Gerven JM. Biomarkers for the effects of cannabis and THC in healthy volunteers. *Br J Clin Pharmacol.* 2009;67:5-21.
16 Dumont GJ, Verkes RJ. A review of acute effects of 3,4-methylenedioxymethamphetamine in healthy volunteers. *J Psychopharmacol.* 2006;20:176-187.
17 Zoethout RW, Delgado WL, Ippel AE, Dahan A, van Gerven JM. Functional biomarkers for the acute effects of alcohol on the central nervous system in healthy volunteers. *Br J Clin Pharmacol.* 2011;71:331-350.
18 Strauss E, Sherman EMS, Spreen O. A Compendium of Neuropsychological Tests; Administration, Norms and Commentary. 3rd ed. New York: Oxford University Press, Inc.; 1998.
19 Lezak MD HDLD. Neurosphychological Assesment. 4th ed. New York: Oxford University Press; 2004.

20 Conners CK, Sitarenios G, Parker JD, Epstein JN. Revision and restandardization of the Conners Teacher Rating Scale (CTRS-R): factor structure, reliability, and criterion validity. *J Abnorm Child Psychol.* 1998;26:279-291.
21 Ullmann RK, Sleator EK, Sprague RL. A new rating scale for diagnosis and monitoring of ADD children. *Psychopharmacol Bull.* 1984;20:160-164.
22 Pliszka S. Practice parameter for the assessment and treatment of children and adolescents with attention-deficit/hyperactivity disorder. *J Am Acad Child Adolesc Psychiatry.* 2007;46:894-921.
23 Farmacotherapeutisch Kompas Nederland. yearly ed. College voor Zorgverzekeringen; 2009.
24 Hinshaw SP, Heller T, McHale JP. Covert antisocial behavior in boys with attention-deficit hyperactivity disorder: external validation and effects of methylphenidate. *J Consult Clin Psychol.* 1992;60:274-281.
25 Pelham WE, Hoza B, Kipp HL, Gnagy EM, Trane ST. Effects of methylphenidate and expectancy of ADHD children's performance, self-evaluations, persistence, and attributions on a cognitive task. *Exp Clin Psychopharmacol.* 1997;5:3-13.
26 Malone MA, Kershner JR, Siegel L. The effects of methylphenidate on levels of processing and laterality in children with attention deficit disorder. *J Abnorm Child Psychol.* 1988;16:379-395.
27 Overtoom CC, Verbaten MN, Kemner C, Kenemans JL, Van EH, Buitelaar JK, et al. Effects of methylphenidate, desipramine, and L-dopa on attention and inhibition in children with Attention Deficit Hyperactivity Disorder. *Behav Brain Res.* 2003;145:7-15.
28 Jonkman LM, Kemner C, Verbaten MN, Van EH, Camfferman G, Buitelaar JK, et al. Attentional capacity, a probe ERP study: differences between children with attention-deficit hyperactivity disorder and normal control children and effects of methylphenidate. *Psychophysiology.* 2000;37:334-346.
29 Jonkman LM, Kemner C, Verbaten MN, Koelega HS, Camfferman G, vd Gaag RJ, et al. Effects of methylphenidate on event-related potentials and performance of attention-deficit hyperactivity disorder children in auditory and visual selective attention tasks. *Biol Psychiatry.* 1997;41:690-702.
30 Klorman R, Salzman LF, Bauer LO, Coons HW, Borgstedt AD, Halpern WI. Effects of two doses of methylphenidate on cross-situational and borderline hyperactive children's evoked potentials. *Electroencephalogr Clin Neurophysiol.* 1983;56:169-185.
31 Michael RL, Klorman R, Salzman LF, Borgstedt AD, Dainer KB. Normalizing effects of methylphenidate on hyperactive children's vigilance performance and evoked potentials. *Psychophysiology.* 1981;18:665-677.
32 Miller DC, Kavcic V, Leslie JE. ERP changes induced by methylphenidate in boys with attention-deficit hyperactivity disorder. *Journal of Attention Disorders.* 1996;1:95-113.
33 Novak GP, Solanto M, Abikoff H. Spatial orienting and focused attention in attention deficit hyperactivity disorder. *Psychophysiology.* 1995;32:546-559.
34 Pliszka SR, Liotti M, Bailey BY, Perez R, III, Glahn D, Semrud-Clikeman M. Electrophysiological effects of stimulant treatment on inhibitory control in children with attention-deficit/hyperactivity disorder. *J Child Adolesc Psychopharmacol.* 2007;17:356-366.
35 Sunohara GA, Malone MA, Rovet J, Humphries T, Roberts W, Taylor MJ. Effect of methylphenidate on attention in children with attention deficit hyperactivity disorder (ADHD): ERP evidence. *Neuropsychopharmacology.* 1999;21:218-228.
36 Jonkman LM, Kemner C, Verbaten MN, Van EH, Kenemans JL, Camfferman G, et al. Perceptual and response interference in children with attention-deficit hyperactivity disorder, and the effects of methylphenidate. *Psychophysiology.* 1999;36:419-429.
37 Verbaten MN, Overtoom CC, Koelega HS, Swaab-Barneveld H, van der Gaag RJ, Buitelaar J, et al. Methylphenidate influences on both early and late ERP waves of ADHD children in a continuous performance test. *J Abnorm Child Psychol.* 1994;22:561-578.
38 Winsberg BG, Javitt DC, Silipo GS. Electrophysiological indices of information processing in methylphenidate responders. *Biol Psychiatry.* 1997;42:434-445.
39 Solanto MV. Dopamine dysfunction in AD/HD: integrating clinical and basic neuroscience research. *Behav Brain Res.* 2002;130:65-71.
40 Volkow ND, Wang GJ, Fowler JS, Ding YS. Imaging the effects of methylphenidate on brain dopamine: new model on its therapeutic actions for attention-deficit/hyperactivity disorder. *Biol Psychiatry.* 2005;57:1410-1415.
41 Barrickman L, Noyes R, Kuperman S, Schumacher E, Verda M. Treatment of ADHD with fluoxetine: a preliminary trial. *J Am Acad Child Adolesc Psychiatry.* 1991;30:762-767.
42 Milberger S, Biederman J, Faraone SV, Chen L, Jones J. ADHD is associated with early initiation of cigarette smoking in children and adolescents. *J Am Acad Child Adolesc Psychiatry.* 1997;36:37-44.
43 Davids E, Zhang K, Tarazi FI, Baldessarini RJ. Animal models of attention-deficit hyperactivity disorder. *Brain Res Brain Res Rev.* 2003;42:1-21.
44 Horner WE, Johnson DE, Schmidt AW, Rollema H. Methylphenidate and atomoxetine increase histamine release in rat prefrontal cortex. *Eur J Pharmacol.* 2007;558:96-97.
45 Wilens TE. Effects of methylphenidate on the catecholaminergic system in attention-deficit/hyperactivity disorder. *J Clin Psychopharmacol.* 2008;28:S46-S53.
46 Prince J. Catecholamine dysfunction in attention-deficit/hyperactivity disorder: an update. *J Clin Psychopharmacol.* 2008;28:S39-S45.
47 Bush G. Attention-deficit/hyperactivity disorder and attention networks. *Neuropsychopharmacology.* 2010;35:278-300.
48 Barry RJ, Johnstone SJ, Clarke AR. A review of electrophysiology in attention-deficit/hyperactivity disorder: II. Event-related potentials. *Clin Neurophysiol.* 2003;114:184-198.
49 Zillessen KE, Scheuerpflug P, Fallgatter AJ, Strik WK, Warnke A. Changes of the brain electrical fields during the continuous performance test in attention-deficit hyperactivity disorder-boys depending on methylphenidate medication. *Clin Neurophysiol.* 2001;112:1166-1173.
50 Kavale K. The efficacy of stimulant drug treatment for hyperactivity: a meta-analysis. *J Learn Disabil.* 1982;15:280-289.
51 Coghill DR, Seth S, Pedroso S, Usala T, Currie J, Gagliano A. Effects of Methylphenidate on Cognitive Functions in Children and Adolescents with Attention-Deficit/Hyperactivity Disorder: Evidence from a Systematic Review and a Meta-Analysis. *Biol Psychiatry.* 2013.
52 Hermens DF, Cooper NJ, Clark CR, Debrota D, Clarke SD, Williams LM. An integrative approach to determine the best behavioral and biological markers of methylphenidate. *J Integr Neurosci.* 2007;6:105-140.
53 Linssen AM, Vuurman EF, Sambeth A, Nave S, Spooren W, Vargas G, et al. Contingent negative variation as a dopaminergic biomarker: evidence from dose-related effects of methylphenidate. *Psychopharmacology (Berl).* 2011;218:533-542.
54 Barkley RA, DuPaul GJ, McMurray MB. Attention deficit disorder with and without hyperactivity: clinical response to three dose levels of methylphenidate. *Pediatrics.* 1991;87:519-531.
55 Beery SH, Quay HC, Pelham WE, Jr. Differential Response to Methylphenidate in Inattentive and Combined Subtype ADHD. *J Atten Disord.* 2013.
56 Grizenko N, Rodrigues Pereira RM, Joober R. Sensitivity of scales to evaluate change in

symptomatology with psychostimulants in different ADHD subtypes. *J Can Acad Child Adolesc Psychiatry.* 2013;22:153-158.
57 Rosenthal R. The File Drawer Problem and Tolerance for Null Results. *Psychological Bulletin.* 1979;86:638-641.
58 Konrad K, Neufang S, Fink GR, Herpertz-Dahlmann B. Long-term effects of methylphenidate on neural networks associated with executive attention in children with ADHD: results from a longitudinal functional MRI study. *J Am Acad Child Adolesc Psychiatry.* 2007;46:1633-1641.
59 Kimko H, Gibiansky E, Gibiansky L, Starr HL, Berwaerts J, Massarella J, et al. Population pharmacodynamic modeling of various extended-release formulations of methylphenidate in children with attention deficit hyperactivity disorder via meta-analysis. *J Pharmacokinet Pharmacodyn.* 2012;39:161-176.
60 Handen BL, Janosky J, McAuliffe S, Breaux AM, Feldman H. Prediction of response to methylphenidate among children with ADHD and mental retardation. *J Am Acad Child Adolesc Psychiatry.* 1994;33:1185-1193.
61 Hermens DF, Cooper NJ, Kohn M, Clarke S, Gordon E. Predicting stimulant medication response in ADHD: evidence from an integrated profile of neuropsychological, psychophysiological and clinical factors. *J Integr Neurosci.* 2005;4:107-121.
62 Greenhill L, Kollins S, Abikoff H, McCracken J, Riddle M, Swanson J, et al. Efficacy and safety of immediate-release methylphenidate treatment for preschoolers with ADHD. *J Am Acad Child Adolesc Psychiatry.* 2006;45:1284-1293.
63 Wigal T, Greenhill L, Chuang S, McGough J, Vitiello B, Skrobala A, et al. Safety and tolerability of methylphenidate in preschool children with ADHD. *J Am Acad Child Adolesc Psychiatry.* 2006;45:1294-1303.
64 Cornforth C, Sonuga-Barke E, Coghill D. Stimulant drug effects on attention deficit/hyperactivity disorder: a review of the effects of age and sex of patients. *Curr Pharm Des.* 2010;16:2424-2433.
65 Cornforth C, Sonuga-Barke E, Coghill D. Stimulant drug effects on attention deficit/hyperactivity disorder: a review of the effects of age and sex of patients. *Curr Pharm Des.* 2010;16:2424-2433.
66 Porrino LJ, Rapoport JL, Behar D, Ismond DR, Bunney WE, Jr. A naturalistic assessment of the motor activity of hyperactive boys. II. Stimulant drug effects. *Arch Gen Psychiatry.* 1983;40:688-693.
67 Kimko H, Gibiansky E, Gibiansky L, Starr HL, Berwaerts J, Massarella J, et al. Population pharmacodynamic modeling of various extended-release formulations of methylphenidate in children with attention deficit hyperactivity disorder via meta-analysis. *J Pharmacokinet Pharmacodyn.* 2012;39:161-176.
68 Swanson J, Gupta S, Guinta D, Flynn D, Agler D, Lerner M, et al. Acute tolerance to methylphenidate in the treatment of attention deficit hyperactivity disorder in children. *Clin Pharmacol Ther.* 1999;66:295-305.
69 Teicher MH, Polcari A, Anderson CM, Andersen SL, Lowen SB, Navalta CP. Rate dependency revisited: understanding the effects of methylphenidate in children with attention deficit hyperactivity disorder. *J Child Adolesc Psychopharmacol.* 2003;13:41-51.
70 Graham J, Coghill D. Adverse effects of pharmacotherapies for attention-deficit hyperactivity disorder: epidemiology, prevention and management. CNS *Drugs.* 2008;22:213-237.
71 Charach A, Ickowicz A, Schachar R. Stimulant treatment over five years: adherence, effectiveness, and adverse effects. *J Am Acad Child Adolesc Psychiatry.* 2004;43:559-567.
72 McCarthy S, Asherson P, Coghill D, Hollis C, Murray M, Potts L, et al. Attention-deficit hyperactivity disorder: treatment discontinuation in adolescents and young adults. *Br J Psychiatry.* 2009;194:273-277.

TABLE 1 **Literature search queries**

Database	Full search query
PubMed	('Attention Deficit Disorder with Hyperactivity'[Mesh] OR 'attention deficit hyperactivity disorder'[all fields] OR 'attention deficit disorder'[all fields] OR 'adhd'[all fields] OR 'minimal brain disfunction'[all fields] OR 'attention-deficit/hyperactivity disorder'[all fields]) AND ('methylphenidate'[Mesh Terms] OR 'methylphenidate' [All Fields] OR ('methylphenidate'[Mesh Terms] OR 'methylphenidate'[All Fields] OR 'dexmethylphenidate' [All Fields]) OR ('methylphenidate'[Mesh Terms] OR 'methylphenidate'[All Fields] OR 'ritalin'[All Fields]) OR 'stimulant medication'[all fields] OR 'stimulant medications'[all fields]) AND ('humans'[Mesh Terms] AND English[lang] AND (Clinical Trial[ptyp] OR Randomized Controlled Trial[ptyp] OR Clinical Conference[ptyp] OR Clinical Trial, Phase I[ptyp] OR Clinical Trial, Phase II[ptyp] OR Clinical Trial, Phase III[ptyp] OR Clinical Trial, Phase IV[ptyp] OR Comparative Study[ptyp] OR Consensus Development Conference[ptyp] OR Consensus Development Conference, NIH[ptyp] OR Evaluation Studies[ptyp] OR Research Support, Non US Gov't[ptyp] OR Research Support, US Gov't, Non PHS[ptyp]))
EMBASE	(*attention deficit disorder/ or attention deficit hyperactivity disorder.ti,ab. or attention deficit disorder. ti,ab. or adhd.ti,ab. or minimal brain disfunction.ti,ab.) AND (methylphenidate.ti,ab. or *methylphenidate/ or dexmethylphenidate.ti,ab. OR Ritalin.ti,ab.) AND (biomarker*.mp. or biological marker/ or biological marker*. mp. OR treatment outcome*.mp. or treatment outcome/ OR Treatment Response*.mp. OR Treatment Effect*. mp. OR predictor*.mp.)
PSYCINFO	(DE 'Attention Deficit Disorder with Hyperactivity' or DE 'Attention Deficit Disorder' or TX ADHD or TX attention deficit disorder or TX attention deficit hyperactivity disorder or TX minimal brain disfunction) AND (DE 'Methylphenidate' OR TX methylphenidate OR TX dexmethylphenidate OR TX Ritalin) AND (DE 'Biological Markers' OR TX biological marker* OR TX biomarker* OR DE 'Treatment Outcomes' or TX treatment outcome* or TX treatment response* OR TX treatment effect* OR TX predictor*

TABLE 2 **Characteristics of studies included in the analysis**

		Tests n (%)	Observations n (%)	Total n (%)
Number of studies		72	31	78
Sex	Male	1769 (91%)	1423 (93%)	2695 (92%)
	Female	168 (9%)	100 (7%)	234 (8%)
MPH dose	High (≥0.60 mg/kg)	26 (36%)	12 (39%)	29 (37%)
	Medium (0.25-0.59 mg/kg)	66 (92%)	28 (90%)	72 (92%)
	Low (0.1-0.25)	15 (21%)	6 (19)	16 (21%)
Wash-out period	> 1 week	9 (13%	0 (0%)	9 (12%
	> 5 half-lives	63 (88%)	31 (100%)	69 (89%)
Age	5-13 years	58 (81%)	27 (87%)	64 (82%)
	<5 or > 13 years	14 (19%)	4 (13%)	14 (18%)
Design	Crossover	66 (92%)	29 (61%)	72 (92%)
	Parallel Group	6 (8%)	2 (7%)	6 (8%)

TABLE 3 Reported test, observation per cluster, and domain

Domain	Cluster	Standardized Names of Tests and Observations	Number of tests (n)	Number of studies (n)
		TESTS	533	
Attention			148	72
	Divided Attention	Arrow/Sound (In)CompatibilityTask, Dichotic Listening Task, Divided Attention Task, Dual Task	7	5
	Focused/Selective Attention	Alertness Task, Arrow/Direction (In)Compatibility Task, Auditory Selective Attention Task (ERP), Children's Checking Task, Find-A-Word Task (nonsense word), (Go/No-)Go Task, Graduated Holes Task (*), Letter Matching Task, Letter Search Task, Matching Familiar Figures Task, Matching to Sample Task (simultaneous), Oddball Task (ERP), Operant Task, Orientation Task (ERP), Puzzle Task, Saccadic eye movements - predictable location/timing, Selective Auditory Attention Task, Stroop Word Color Task, Tachistoscopic Task, Visual Memory Search Task, Visual Scanning Task, Visual Selective Attention Task (ERP), Visual Spatial Focused Attention Task (ERP)	54	27
	Reaction Time	Eriksen Flanker Task (*), Choice Reaction Time Task, Flexibility Task(*), Go/No-Go Task, Oddball Task (*), Reaction Time Task, Saccadic eye movements - visually guided (*), Set Shifting Task (*), Stroop Word Color Task (*)	37	15
	Sustained Attention (Vigilance)	Academic Task Performance, Arithmetic Task, Eriksen Flanker Task (ERP), Auditory Selective Attention Task, Card Sorting Task, Change Task, Continuous Performance Task (ERP), Find-A-Word Task - common words, Find-A-Word Task - nonsense word, Easy/Hard Decision Task (ERP), Letter Matching Task, Oddball Task, Puzzle Task*, Set Shifting Task*, Timed Reading Task*, Vigilance Task, Visual Selective Attention Task, Word discovery Task	50	25
Behavior			2	1
	Aggression	Social Information Processing	2	1
Executive			243	66
	Creativity	Alternate Uses Test, Narrative Discourse/Task*	4	2
	Impulsivity	Arrow/Direction (In)Compatibility Task*, Arrow/Sound (In)CompatibilityTask*, Auditory Selective Attention Task*, Cambridge Gamble Task, Change Task, Circle Tracing Task, Continuous Performance Task*, Divided Attention Task*, Eriksen Flanker Task, Follow Task, Go/No-Go Task (ERP), Information Sampling Task, Matching Familiar Figures Task*, Oddball Task*, Operant Task*, Orientation Task*, Reaction Time Task*, Saccadic eye movements - anti saccade, Set Shifting Task, Stroop Word Color Task, Tower of London Task*, Vigilance Task*, Visual Scanning Task*, Visual Selective Attention Task*, Visual Selective Attention Task*, Visual Spatial Focused Attention Task*	86	29
	Motivation	Find-A-Word Task - nonsense word, Persuasive Persistence Task	5	2
	Planning	Stockings of Cambridge, Tower of London Task	6	3
	Reasoning/ Association/ Problem Solving	Academic Task Performance, Arithmetic Task, Classroom Observation, Listening Comprehension Task, Narrative Discourse/ Task*, Spelling Task, Story Retelling Task	42	12
Executive	Set Shifting	Alternate Uses Test, Card Sorting Task, Change Task, Flexibility Task, Set Shifting Task	14	7
	Spatial Orientation	Visual Spatial Processing Task	1	1
	Time/Distance Estimation	Sensorimotor Anticipation Task, Time Discrimination Task	2	1
	Working Memory/ Immediate Recognition	Arithmetic Task (mental), Digit Span - Backward, Digit Span - Forward, Dual Task*, Matching to Sample Task (delayed), Pattern Recognition Task, Spatial Recognition Memory Task, Spatial Span - Backward, Spatial Span - Forward, Spatial Span Task, Spatial Working Memory Task, Spatial Working Memory Task - Backward, Spatial Working Memory Task - Forward	83	9
Language			39	16
	Comprehension	Listening Comprehension Task, Reading Comprehension Task, Story Retelling Task	6	3
	Production	Alternate Uses Test, Digit Naming Task, Instances test, Letter Naming Task, Naming Task, Narrative Discourse/ Task, Rhyming Task, Story Retelling Task, Word discovery Task	18	6
	Spelling/Grammar/ Semantics	Color Naming Task, Mother-Child Language, Sequence Insertion Task, Spelling Task, Tachistoscopic Task, Timed Reading Task, Trigram Naming Task	15	7
Memory			27	14
	Auditory/Verbal Memory: Delayed Recall	Paired Associate Learning Task, Spelling Task	5	2
	Auditory/Verbal Memory: Delayed Recognition	Listening Comprehension Task, Verbal Memory Task	4	2
	Auditory/ Verbal Memory: Immediate Recall	Letter Matching Task*, Social Information Processing, Story Retelling Task, Verbal Memory Task	8	4
	Learning	Paired Associate Learning Task, Spelling Task	8	4
	Visual/Spatial Memory: Delayed Recognition	Visual Memory Task	1	1
	Visual/Spatial Memory: Immediate Recall	Visual Memory Task	1	1
Motor			42	11
	Postural Stability	Postural Stability Task	1	1
	Motor Control	Activity level, Finger Tapping, Graduated Holes Task, Softball Performance (sports), Usual Walking Task	32	8
	Visuo-Motor Control	Pointing Task, Visual Motor Integration Task	9	2

Perception			3	1
	Visual Perception	Tachistoscopic Task	3	1
Neurophysiologic			32	14
	EEG	EEG - CPT, EEG - eyes open	8	2
	Evoked Potential	Auditory Evoked Potential, Visual Evoked Potential	23	11
	Eye Movements - Saccadic	Saccadic eye movements - visually guided/(anti)saccadic mixed	1	1
	MEG	Magnetoencephalography	1	1
Subjective Experience			2	1
	Empathy	Story Retelling Task	2	1
(Neuro) Endocrine			2	2
	Prolactin	Prolactin determination	1	1
	Serotonin	5-HIAA determination	1	1
		OBSERVATIONS	232	69
Attention			30	13
	Scale - Attention	ADD-h Comprehensive Teacher Rating Scale (ACTeRS) (P), Classroom Observation (R), On-task Behavior Observation (R/T), Profile of Mood States (S), Softball Performance (sports) (R), Swanson, Nolan and Pelham Questionnaire (SNAP-IV) (P), VAS Alertness (S)	30	13
Behavior			59	14
	Scale - Aggression	Oppositional Behavior Observation (R), Profile of Mood States (S)	4	2
	Scale - Anxiety	Profile of Mood States (S)	2	1
	Scale - Compulsive/ Obsessive	Clinical symptoms of perseveration (R)	4	1
	Scale - Oppositional	Behavior after Failure (R/S); Behavior after Success (R/S); Conners, Loney and Milich Scale (CLAM) (T); Daily Report Card System (R); Following Rules Observation (R/T); IOWA Conners Rating Scale (R); IOWA Conners Teachers Rating Scale (T); Oppositional Behavior Observations (R/T)	49	10

Disease Intensity			41	9
	Scale - ADHD	Abbreviated Conners Teacher Rating Scale (T), Behavior Evaluation (S), Behavior Observation (R), Conners Global Index (R), Conners, Loney and Milich Scale (CLAM) (T), Daily Report Card System (R), IOWA Conners Rating Scale (R), IOWA Conners Teachers Rating Scale (T), UC-Conners Child Behavior Scale (R)	41	9
Executive			41	12
	Scale - Impulsivity	ADD-h Comprehensive Teacher Rating Scale (ACTeRS) (P), Clinical Symptoms of Perseveration (R), Conners Revised Teacher Rating Scale (T), Conners Revised Teacher Rating Scale (T), Impulsivity Behavior Observation (R), Swanson, Nolan and Pelham Questionnaire (SNAP-IV) (P)	33	9
	Scale - Motivation	Behavior after Failure (S/R), Behavior after Success (S/R), Effort Rating (R), Math Cheating Task (R), Success/Failure Puzzle Task (R)	8	3
Social behavior			32	8
	Scale - Social Interaction	Social Behavior Observation (R/T), Social Information Processing (S/R)	32	8
Subjective Experience			29	13
	Scale - Calmness	VAS Calmness (S)	1	1
	Scale - Confusion	Profile of Mood States (S)	2	1
	Scale - Fatigue	Profile of Mood States (S)	2	1
	Scale - Mood	Mood Evaluation (S), Profile of Mood States (S), UC-Conners Child Behavior Scale (R), VAS Happiness (S)	8	4
	Scale - Performance	Behavior after Failure (S), Behavior after Success (S), Performance Prediction (S), Self-Evaluation (S), Success/Failure Puzzle Task (S)	10	4
	Scale - Drug Effect	Attribution Evaluation (S), Success/Failure Puzzle Task (S)	6	2

(ERP), indicates tasks that were used to measure the effect of MPH on attention of impulsivity using ERP.
Subjective measure could be rated by a Researcher (R), a teacher (T), a Parent (P) or the subject (S).
*, indicates a secondary parameter of the task.

TABLE 4 Improvement following MPH for each individual test or observation that was used in at least five studies

	Test/Observation	Number of tests with improvement (n)	Total of tests (n)	Impro-vement (% of tests)	Studies (n)
Test	Go Task	7	11	63.6	7
	Reaction Time Task	9	18	50.0	6
	Continuous Performance Task	16	21	76.2	9
	Go/No-Go Task	13	21	61.9	10
	Arithmetic Task	11	28	39.3	6
	Visual Evoked Potential	16	19	84.2	10
Observation	Following Rules Observation	15	15	100.0	5
	Oppositional Behavior Observations	12	14	85.7	6
	On-task Behavior Observation	12	12	100.0	7
	Impulsivity Behavior Observation	13	19	68.4	6
	Social Behavior Observation	12	32	37.5	6

TABLE 5 Effect of MPH for each functional cluster that was used across at least five studies

Domain	Cluster	Test/ Observation	Improvement (%)			Tests (n)	Studies (n)
			(-)	(=)	(+)		
Attention	Divided Attention	Test	-	28.6	71.4	7	5
	Focused/Selective Attention	Test	1.9	54.7	43.4	54	26
	Reaction Time	Test	-	50.0	50.0	28	9
	Reaction Time*	Test*	-	25.0	75.0	8	6
	Sustained Attention (Vigilance)	ERP	-	60.0	40.0	10	6
	Sustained Attention (Vigilance)	Test	-	23.9	76.1	50	22
	Scale - Attention	Observation	-	16.7	83.3	30	13
Behavior	Scale - Oppositional	Observation	2.0	12.2	85.7	49	10
Disease Intensity	Scale - ADHD	Observation	-	19.5	80.5	41	9
Executive	Impulsivity	Test	-	50.0	50.0	46	16
	Impulsivity*	Test*	-	43.6	56.4	39	13
	Reasoning/Association/ Problem Solving	Test	-	61.0	39.0	41	11
	Set Shifting	Test	-	57.1	42.9	14	7
	Working Memory/ Immediate Recognition	Test	-	69.5	30.5	82	8
Language	Production	Test	-	83.3	16.7	18	6
	Spelling/Grammar/ Semantics	Test	6.7	46.7	46.7	15	7
	Scale - Impulsivity	Observation	-	21.2	78.8	33	9
Motor	Motor Control	Test	-	28.1	71.9	32	8
Neuro-physiologic	Evoked Potential	Test	-	13.0	87.0	23	11
Social behavior	Scale - Social Interaction	Observation	-	64.9	35.1	32	8

'+' reflects an improvement or increase, '=' reflects no significant effect and '-' reflects an impairment or decrease as measured by the corresponding test. Whenever tests provided different parameters with information on more than one functional domain, effects were scored separately, and the secondary effects were marked (*).

TABLE 6 Dose-response relationship for outcome measures and functional clusters that showed a consistent MPH response in a reasonable number of studies

Domain, cluster and/or test	Test/ Observation	Low Dose				Medium Dose				High Dose			
		(-)	(=)	(+)	n	(-)	(=)	(+)	n	(-)	(=)	(+)	n
Attention													
Sustained Attention (Vigilance)	Test	-	100	0	2	-	28	72	22	-	11	89	6
Motor													
Motor Control	Test	-	33	67	5	-	33	67	5	-	18	82	3
Disease Intensity													
Scale - ADHD	Observation	-	11	89	4	-	27	73	9	-	0	100	4
Individual test													
Go/No-go Task	Test	-	20	80	5	-	50	50	6	-	25	75	3

n is the number of studies in which the cluster was evaluated (for each dose)

CHAPTER 4

Determination of methylphenidate in plasma and saliva by liquid chromatography/tandem mass spectrometry

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ABSTRACT

Methylphenidate (MPH) is a phenethylamine derivative used in the treatment of attention-deficit hyperactivity disorder (ADHD). In adults, clinical monitoring of MPH therapy is usually performed by measuring plasma MPH concentrations. In children blood sampling is however undesirable. Saliva may be an alternative matrix for monitoring MPH concentrations with the advantage that it can be obtained non-invasively. Therefore, we developed an analytical method for the quantification of MPH in both plasma and saliva.
We present the validation of a liquid chromatography-tandem mass spectrometric method using a hydrophilic interaction liquid chromatography column (HILIC). In 100 µl sample, proteins were precipitated with 750 µl acetonitrile/methanol 84/16 (v/v) containing d_9-methylphenidate as the internal standard. Standard curves were prepared over the MPH concentration range of 0.5-100.0 µg/L. The total analysis time was 45 seconds. Accuracy and within- and between-run imprecision were in the range of 98-108% and less than 7.0%, respectively. Matrix effects were greater for plasma than saliva with 46% and 8% ionization suppression. The matrix effects were adequately compensated by the use of deuterated MPH as internal standard. MPH significantly degraded in plasma and saliva at room temperature and 5°C. Samples were stable at -20°C for at least 4 weeks. The method was successfully applied for the determination of MPH concentrations in plasma and saliva samples from an adult healthy volunteer. Using protein precipitation and hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry, this method allows fast, accurate and precise quantification of MPH in both plasma and saliva.

Introduction

Methylphenidate (MPH) is a psychostimulant widely used in the treatment of attention-deficit hyperactivity disorder (ADHD). ADHD is a neurobehavioral problem mostly encountered in school-aged children at a prevalence of 5-10% of the general population[1,2]. MPH is a piperidine-derived molecule that contains two chiral centers and exists as four stereoisomers (Figure 1). The pharmacological activity resides entirely with the *dl-threo*-methylphenidate racemic (50:50) mixture[3].

The major metabolic pathway of MPH is the hydrolysis of the methyl ester linkage by esterases to form ritalinic acid[4–5]. Minor metabolic pathways for both these compounds include parahydroxylation of the aromatic ring, oxidation to 6-oxo-dervatives and glucuronide formation[6–7]. Ritalinic acid and the other metabolites are pharmacologically inactive[8–10].

There is a clinical need to perform therapeutic drug monitoring (TDM) in patients who are undergoing MPH therapy. MPH exhibits wide inter-individual variability in both pharmacokinetics and clinical response[11–12]. TDM can be applied when the patient remains unresponsive to therapy, exhibits unexpected adverse events or to check adherence. In adults, clinical monitoring of MPH therapy is usually performed by measuring plasma MPH concentrations. In children, monitoring of drug levels should be performed with minimal discomfort for the patient. Monitoring of MPH concentrations in saliva may therefore be an interesting non-invasive alternative to blood sampling as demonstrated earlier by Marchei and coworkers[13].

Several methods have been developed for quantification of MPH in plasma, urine and hair, using high-performance liquid chromatography (HPLC) with ultraviolet detection[6,14], capillary electrophoresis-mass spectrometry[15], gas chromatography-mass spectrometry[8,9,16] and liquid chromatography–tandem mass spectrometry[7,13,17–18]. The determination of MPH concentrations by standard reversed-phase (RP) chromatography coupled to MS/MS detection is particularly challenging since retention times may be short due to the high hydrophilicity of the compound. This may produce a significant loss in sensitivity due to the co-elution with matrix interference and the high percentage of water at the chromatographic elution time. Recent research however has shown that for hydrophilic compounds the sensitivity, precision and accuracy of a quantitative analytical chromatographic method may be improved by using hydrophilic interaction liquid chromatography (HILIC)[19]. In addition, the use of HILIC has advantages in sample preparation when measuring polar compounds. Because of the high organic modifier content, usually acetonitrile, used during chromatography, proteins can be precipitated using organic solvents without the loss of chromatographic integrity, as is often the case when used with polar compounds in combination with RP chromatography. A high organic modifier concentration is also ideal for compound ionization by electrospray ionization mass spectrometry (ESI-MS), resulting in higher sensitivity.

The aim of the present study was to develop a method to determine MPH concentrations in human plasma and saliva for potential use in therapeutic drug monitoring. We present the development and validation of an analytical method using HILIC chromatography coupled to tandem mass spectrometry. The stability of MPH in plasma and saliva was investigated at different temperatures. The applicability of the method was demonstrated with plasma and saliva data from one healthy adult volunteer obtained before and after intake of 10 mg immediate release (IR) MPH and 18 mg MPH – osmotic controlled-release oral delivery system (OROS) – on different occasions.

Materials and methods

Chemicals

Methylphenidate was purchased from Bufa (Uitgeest, Netherlands). As an internal standard (I.S.) a 1 mg/ml solution of deuterated methylphenidate HCl (d_9-MPH) in methanol was obtained from LGC-Standards (Teddington, United

Kingdom) (Figure 1). Water was purified and deionized using an ELGA purelab Optron Q (Veolia Water; Saint Maurice, France). Drug free, non sterile, K_2 EDTA human plasma was obtained from Equitech-Bio (Kerrville-TX, USA). OraFlx synthetic saliva was obtained from Dyna-Tek (Lenexa-KS, USA).

Instrumentation

The LC-MS setup comprised of a Thermo Scientific Surveyor LC (Waltham-MA, USA) system coupled to a Maylab Mistraswitch column oven (Spark Holland, Netherlands Emmen), and a Thermo Scientific TSQ Quantum Access MS system with an ESI source. The Xcalibur 2.0.7 SP1 (Thermo Scientific) software package was used for controlling the LC-MS system and for data processing.

LC-MS/MS *conditions*

Isocratic elution was applied using A: 2% formic acid in water (v/v) and B: acetonitrile 100%. A was set at 10% and B at 90%. Analytical separation was accomplished on a SeQuant ZIC-HILIC column (Merck, Darmstadt, Germany) with a length of 50 mm, an internal diameter of 2.1 mm and 5 µm particle size. The flow rate was 1.00 ml/min giving a total chromatographic run time of only 45 seconds. To minimize carry-over effects the LC injection system was washed with 20% formic acid in water (v/v) after every injection. The autosampler temperature was maintained at 10°C, the column oven at 30°C. The analytes were detected in positive ion mode using multiple reaction monitoring (MRM). The ion spray voltage was 5000 V and the ion transfer tube temperature was 250°C. Sheath and auxiliary gas pressure were 50 and 20 psi, respectively. Collision gas (argon) pressure was 2.0 mTorr. MPH and d_9-MPH were measured as $[M+H]^+$ using the mass transitions 234.1 → 84.1 and 243.1 → 93.1 respectively. Tube lens voltage and collision energy were 90 and 21 V, respectively. Dwell time was 300 ms for MPH and 50 ms for d_9-MPH.

Analytical procedures

PREPARATION OF STOCK SOLUTIONS, CALIBRATION STANDARDS AND QUALITY CONTROL SOLUTIONS

Two stock MPH solutions (5.0 mg/L and 50.0 mg/L) were prepared by dissolving MPH in water/methanol 1/1 (v/v). The stock internal standard (10 µg/L) was prepared by diluting the 1 mg/ml d_9-MPH standard solution with acetonitrile/methanol 84/16 (v/v). All stock solutions were stored at 5°C until use.

Four MPH calibration standard solutions (5.0, 10.0, 25.0 and 50.0 µg/L) were prepared by diluting 10, 20, 50, and 100 µl of the 5.0 mg/L stock solution in 10 ml of water/methanol 1/1 (v/v). Three MPH calibration standard solutions (100, 400 and 1000 µg/L) were prepared by diluting 20, 80 and 200 µl of the 50.0 mg/L stock solution in 10 ml of water/methanol 1/1 (v/v). Quality control (QC) solutions were prepared in a similar manner as the calibration standard solutions. The MPH concentrations of the QC solutions were 5.0, 100 and 1000 µg/L. The calibration standard and QC solutions were stored at 5°C until use.

SAMPLE PREPARATION

Calibration standards and QC samples were prepared just prior to analysis. Calibration standard and QC solutions were shortly vortexed and a volume of 10 µl was pipetted into a 1.8 ml vial. Subsequently, 100 µl saliva or plasma, depending on the composition of the calibration line, was added and shortly vortexed. Final concentrations of the plasma and saliva calibration line were 0.5, 1.0, 2.5, 5.0, 10.0, 40.0 and 100.0 µg/L and final concentrations of the quality controls were 0.5 (QC1 (LLOQ)), 10.0 (QC2) and 100.0 µg/L (QC3). Patient plasma and saliva samples were thawed and shortly vortexed and 100 µl of each sample was pipetted into a 1.8 ml vial. Subsequently, 10 µl of water/methanol 1/1 (v/v) was added and shortly vortexed. In all samples proteins were precipitated by adding 750 µl of the internal standard solution. After vortexing for 1 minute,

samples were stored at -20°C for 30 minutes to enhance protein precipitation, vortexed again and centrifuged for 5 minutes at 480x*g*. Two microliter of the supernatant was injected.

QUANTIFICATION

MS response was expressed as integrated area of the chromatographic peak. For calibration, the concentration of prepared calibration standards was the known variable (x), the ratio of analyte MS response divided by internal standard MS response per calibration level was the unknown variable (y). Patient samples were back-calculated using the calibration line by their respective ratio of analyte / internal standard MS response.

Method validation

SELECTIVITY

One lot of blank, commercially acquired saliva and plasma, together with saliva and plasma samples from five different patients, not receiving MPH, were tested for interferences. Proteins were precipitated using acetonitrile/methanol 84/16 (v/v) without I.S. The data of the chromatograms were processed and the integrated response should not exceed 10% of the average integrated response of the LLOQ of MPH and 1% of the integrated response of d_9-MPH.

CALIBRATION

A total of six calibration lines, consisting of seven different concentrations, were prepared in commercially acquired saliva and plasma and measured during six runs. Calibration curves were obtained by fitting the peak area ratios to a weighted (1/x) least squares regression model.

ACCURACY AND IMPRECISION

The accuracy and imprecision of the method were determined for the QC samples during six consecutive runs. In the first run all QC concentration levels were analyzed in six fold (within-run imprecision); during the following five runs a single sample of each level was analyzed (between-run imprecision). Mean accuracy and within-run imprecision (coefficient of variation) were calculated from the results (n=6) of the first run. Between-run imprecision was calculated from the results (n=6) of the first sample of the first run and the samples of run two through six. According to the US Food and Drug Adminstration guideline for bio-analytical method validation the mean accuracy should be within 85-115% and the within-run and between-run imprecision should be less than 15%[19]. Furthermore, the limit of quantification of the assay was defined as the lowest concentration of MPH that could be detected with a mean accuracy within 80-120% and within-run and between-run imprecision not exceeding 20% of the coefficient of variation[20].

Since plasma and saliva may be diluted to obtain concentrations in the calibration range, the accuracy of diluted samples was determined as well. Plasma and saliva samples were prepared with concentrations of 100 µg/L (QC3) and 1000 µg/L. All samples were diluted ten times with commercially acquired saliva and plasma (10 µl sample + 90 µl plasma/saliva) in six fold and the accuracy was determined. Mean accuracy of the diluted samples should be within 85%-115% and imprecision should be less than 15%.

PROCESS EFFICIENCY AND MATRIX EFFECTS

Plasma, saliva and solvent components in the ionization chamber may cause batch specific ion suppression or enhancement, leading to inter-patient and intra-patient signal variability[21-22]. Assay recovery and matrix effects were quantified for both plasma and saliva using the strategies proposed by Matuszewski *et al*[23]. In short, chromatograms were obtained from plasma and saliva samples that were spiked pre-precipitation, plasma and saliva samples spiked post-precipitation and spiked aqueous solutions. In total, six batches of plasma and saliva were spiked in duplicate; the MPH and d_9-MPH concentrations were 10 µg/L. Recovery (RE) was defined as the relative signal of samples spiked post-precipitation versus pre-precipitation. Matrix effects (ME) were similarly defined as the relative signal of post-precipitation spiked plasma and saliva samples versus spiked aqueous samples. A value of 100% for ME

indicated that signals in plasma/saliva samples and aqueous samples phase were similar. A ME value greater than 100% indicated ionization enhancement, whereas a value less than 100% indicates ionization suppression.

Process efficiency (PE) was defined as the product of RE and ME, i.e. the overall signal of spiked plasma and saliva versus an aqueous standard solution. Average values and coefficients of variation of RE, ME and PE were calculated over the six plasma and saliva batches.

STABILITY

The stability of MPH in saliva and plasma QC1 (LLOQ) and QC3 samples was determined for several storage conditions. The freeze-thaw stability in plasma and saliva was determined by comparing freshly prepared samples with samples that underwent three freeze-thaw cycles (24 hours at -80°C). The MPH concentration of plasma and saliva samples stored at -20°C and -80°C was determined weekly and compared with freshly prepared samples. The stability of MPH in plasma and saliva at 5°C was assessed after 2, 5 and 7 days of storage. The time course of MPH degradation in plasma and saliva was studied at room temperature by determination of the MPH concentration at the start of the experiment and 1, 4, 8, 21.5, 24 and 48 hours after the start. The esterase mediated decay of MPH in plasma and saliva was described by a first-order process. Data were log-transformed and rate constants were obtained by linear regression. Half life was calculated by dividing 0.693 by the rate constant.

The MPH concentration of processed samples stored in the autosampler (10°C) was determined after 24 hours and compared with the initial concentration. The analyte was considered stable in the biological matrix or extracts if 80%-120% (QC1 (LLOQ)) or 85%-115% (QC3) of the reference concentration was recovered.

All stability experiments were performed in triplicate and results were expressed as mean ± SD.

Clinical application

The developed assay was applied to saliva and plasma samples from a healthy adult volunteer participating in a pharmacokinetic study. The study was approved by the local Institutional Ethics Committee. Saliva and blood samples were collected at t=-30, -15 (saliva only), 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360 minutes following the intake of 10 mg MPH-IR (Ritalin). Following ingestion of 18 mg MPH-OROS (Concerta), samples were collected at t=-30, -15 (saliva only), 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 600 and 720 minutes. Study days were separated by at least 5 days to ensure complete wash-out. Blood samples were collected in EDTA tubes and put on ice immediately for 30 min. Saliva samples were obtained using the polyester Salivette swab system (Sarstedt AG, Nümbrecht, Germany). Samples were directly centrifuged at 2000x*g* for 10 minutes at 4 °C and the plasma and saliva were stored at -80°C until analysis.

Results and discussion

Chromatography

Using electrospray in the positive mode MS parameters were tuned to produce maximum responses for MPH and the internal standard d_9-MPH. The protonated molecular ions $[M+H]^+$ were *m/z* 234.1 and 243.1, respectively. The MS2 spectra of both methylphenidate and d_9-methylphenidate are given in Figure 2; the corresponding most abundant product ions were *m/z* 84.1 and 93.1.
The chromatographic results after injection of drug free plasma and saliva, a LLOQ sample and a patient receiving MPH are shown in Figure 3. The chromatography shows excellent peak shape and symmetry, with a peak baseline

resolution of less than 10 seconds. Under the chromatographic conditions employed, the retention times were 24 s for both MPH and internal standard d_9-MPH. The total runtime was 45 s. The reproducibility of the retention times was good for the several columns used during the development and validation of the method and the analysis of several thousand clinical samples (data not shown). The lifetime of the column was acceptable; more than 1000 injections could be made before chromatographic performance became unacceptable.

Total runtimes of reversed-phase chromatographic MS/MS methods for MPH quantification have been reported to range from 5 to 15 min[18, 24-25]. When using reversed phase chromatography, a short retention time of MPH may be unfavorable since sensitivity may be reduced due to co-elution of matrix components. In the present study application of the HILIC column allowed a total runtime of 45 s, which was at least 6-fold shorter than published reversed-phase methods[18, 24-25].

Methods have been developed for the simultaneous determination of MPH and its inactive metabolite ritalinic acid[18, 24-25]. Assesment of the latter may be particularly useful in forensic studies or compliance studies since ritalinic acid concentrations are generally 10- to 50- fold higher than methylphenidate concentrations and RA may be detected longer following ingestion due to its longer elimination half-life[26]. In case of therapeutic drug monitoring detection of ritalinic acid has no value, since the individual dose is adjusted on basis of determined concentrations of methylphenidate only.

Validation

SELECTIVITY

There were no discernable interfering components in commercially available and patient plasma and saliva. Figure 3 shows chromatograms from blank plasma and saliva, plasma and saliva spiked with MPH at LLOQ and d_9-MPH and a patient sample.

CALIBRATION

The calibration curves provided a linear response for the interval 0.5 – 100.0 µg/L. Un-weighted and weighted linear regression 1/x and 1/x2 were compared by means of statistical and graphical methods. A weighting factor of 1/x provided the best fit. The value of each calibration standard was within 90-110% of the nominal value. The correlation coefficients (r^2) of the 1/x-weighted calibration curves were in the range of 0.9997-1.0000 (n=6, mean 0.9999) for plasma and in the range 0.9995-1.0000 (n=6, mean 0.9980) for saliva. The standard curves were y = 0.00791 (0.00130) x + 0.00235 (0.00306) for plasma and y = 0.00807 (0.00130) x + 0.00051 (0.00043) for saliva (mean (95% CI); n=6). For plasma the intercepts with the y-axis was not significantly different from zero, whereas a small but constant error was present for saliva.

ACCURACY AND IMPRECISION

The lower limit of quantification (LLOQ) for MPH was arbitrarily set at 0.5 µg/L (=QC1) in both plasma and saliva. Only noise was detected when blank plasma and saliva samples were injected following the injection of the highest calibration standard; carry-over was less than 0.1%.

The mean accuracy in both saliva and plasma was within the acceptance criteria of 85 – 115% for all QC levels (Table 1). For both plasma and saliva within-day and between-day imprecision were acceptable with values less than 7.0% in all QC samples. The mean accuracy of ten times diluted samples was acceptable as well. Accuracy was 110.5% and 100.2% for plasma samples with a concentration of 100 µg/L (QC3) and 1000 µg/L, respectively; the mean accuracy in saliva was 105.0% and 102.2% at similar concentrations.

PROCESS-EFFICIENCY AND MATRIX EFFECTS

The process efficiency of the used method for the quantification of MPH in plasma was influenced by the occurrence of matrix effects. The matrix effects determined at plasma and saliva concentration of 10 µg/L were 53.9 ± 8.7% and

92.5 ± 10.2% (mean ± SD, n=6), respectively, corresponding to 46.1% and 7.5% ion suppression. Matrix effects for d_9-MPH were comparable: 55.7 ± 11.2% for plasma and 98.0 ± 12.2% for saliva. Notably, the matrix effect for d_9-MPH in plasma was comparable, indicating the beneficial effect of using a deuterated internal standard. Recovery of MPH was 116.6 ± 6.3% in plasma and 103.4 ± 6.6% in saliva; corresponding values for d_9-MPH were 113.8 ± 7.7% and 99.8 ± 7.7% (mean ± SD, n=6). Wang and colleagues reported that a slight difference in retention time between analyte and an internal standard labeled with a stable isotope may cause a different degree of ion suppression between the analogues and, consequently, influence the accuracy of the method[27]. In our method retention times of MPH and d_9-MPH were similar and >90% of the peak areas were overlapping. As a result, patient- and time-dependent variability in ion suppression will not affect the accuracy of our method.

STABILITY

At -80°C MPH concentrations in plasma decreased with 6.4 ± 3.1% (QC1 (LLOQ)) and 2.2 ± 1.9% (QC3) after having been stored for 4 weeks (mean ± SD, n=3). The corresponding values in saliva were 2.4 ± 1.0% and 0.3 ± 0.7%. Degradation at 4 weeks at -20°C was 7.3 ± 4.2% for plasma and 7.8 ± 2.8% for saliva in QC1; corresponding values for QC3 were 2.4 ± 3.4% and 0.3 ± 0.5%. At -80°C and -20°C, stability was not studied for more than 4 weeks of storage. In QC1 plasma and saliva samples stored at 5°C, 43.8 ± 6.1% and 42.7 ± 3.1% was degraded after two days, respectively. In QC3 plasma and saliva samples decay was 39.1 ± 2.7% and 53.0 ± 3.7%. Apparently, degradation of MPH, caused by the catalytic activity of esterases, is still present at 5°C.

Figure 4 presents the degradation profile of MPH in plasma and saliva for QC1 and QC3 at room temperature. After 8 hours of storage the MPH concentration of MPH was decreased with 5.4 ± 4.2% and 7.0 ± 4.9% (mean ± SD, n=3) in the QC1 and QC3 plasma samples, respectively. After 1 hour saliva concentrations were reduced with 13.2 ± 3.5% and 6.9 ± 1.7%, respectively. Half-life in plasma was 81 ± 7h and 68 ± 6h for QC1 and QC3, respectively; corresponding saliva values were 24 ± 4h and 23 ± 3h (mean ± SD, n=3).

The degradation of QC3 plasma and saliva samples after 3 freeze/thaw cycles was acceptable; corresponding values were 6.4 ± 4.2% and 12.4 ± 9.3% (mean ± SD, n=3). For QC1 samples significant degradation was observed in plasma after the second cycle (38.4 ± 9.2%) and in saliva after the third cycle (18.4 ± 6.2%). This indicates at lower concentrations the number of freeze/thaw cycles should be limited to 1.

The processed plasma and saliva samples were stable in the autosampler (10°C) for 24 hours, suggesting that all esterase activity is eliminated after protein precipitation.

Little information is available in literature on the stability of MPH in plasma and saliva. Considering the present results, plasma and saliva samples should be immediately frozen at -20°C after collection from the patient. Protein precipitation should be performed directly following thawing of the sample.

Clinical application

The developed method was successfully applied for the assessment of MPH concentration profiles in plasma and saliva in an adult healthy volunteer taking 10 mg MPH as IR preparation and 18 mg as OROS (Figure 5). The time profile of MPH concentration in saliva followed more or less the plasma time profile. During the first hour after ingestion of the IR preparation, saliva concentrations were approximately two-fold higher than plasma concentrations. This may be caused by some degree of dissolution of the IR formulation in the mouth, as this was given in its commercially available tablet form. Two-fold higher saliva concentrations were also observed following ingestion of the OROS formulation, which is a capsule, indicating that another mechanism influencing the distribution between plasma and saliva may be involved as well. MPH is an ampetamine-like compound that has low plasma protein binding (approximately 15%), and low molecular weight (233 Da) and shows the characteristics of a weak base (pK_a = 8.9). Based on these characteristics, ion trapping may occur, as has also been described for methylenedioxymethamphetamine (MDMA)[28]. The free MPH fraction passively distributes in its ionized

form from blood to saliva (which is more acidic than blood) and then cannot diffuse back into plasma, leading to higher MPH concentrations in saliva compared to those in plasma.

Alternative biological matrices (hair, oral fluid, sweat) have been studied earlier for monitoring therapeutic use or misuse of methylphenidate[18, 29]. Marchei and coworkers demonstrated that the overall patterns of concentration-time profiles of plasma and saliva MPH agreed reasonably well following the administration of fast- and extended-release formulations[13]. They reported higher saliva concentrations than plasma concentrations, which is in accordance with the result of the present study. In the referred study however the saliva/plasma ratio proved to be time- and formulation dependent, which may have hampered clinical application of saliva monitoring so far.

Conclusion

An LC-MS/MS method using hydrophilic interaction liquid chromatography has been successfully developed for determination of MPH concentrations in plasma and saliva. The method has proven to be rapid, sensitive, accurate and precise. Due to matrix effects of plasma, the use of deuterated MPH as an internal standard was essential. Stability experiments demonstrated that samples should be stored at temperatures of -20°C or below directly after sampling, and that samples should be processed immediately after thawing. The assay allows further investigation of therapeutic monitoring of MPH concentrations in saliva.

REFERENCES

1 D.P. Cantwell, *J. Am. Acad. Child. Adolesc. Psychiatry* 35 (1996) 978.
2 J.M. Swanson, J.A. Sergeant, E. Taylor, E.J.S. Sonuga-Barke, P.S. Jensen, D.P. Cantwell, *Lancet* 351(1998) 429.
3 D.J. Heal, D.M. Pierce, *cns Drugs* 20 (2009) 713.
4 N. Srinivas, J. Hubbard, E. Korchinski, K. Midha, *J. Pharm. Sci.* 81 (1992) 747.
5 N. Srinivas, J. Hubbard, D. Quinn, K. Midha, *Clin. Pharmacol. Ther*. 52 (1992) 561.
6 S.J. Soldin, Y.P. Chan, B.M. Hill, J.M. Swanson, *Clin. Chem.* 25 (1979) 401.
7 J.S. Markowitz, C.L. DeVane, D.W. Boulton, Z. Nahas, S.C. Risch, F. Diamond, K.S. Patrick, *Drug Metab. Disp.* 28 (2000) 620.
8 J. Eichhorst, M. Etter, J. Lepage, D.C. Lehotay, *Clin. Biochem.* 37 (2004) 175.
9 S.N. Lin, D.M. Andrenyak, D.E. Moody, R.L. Foltz, *J. Anal. Toxicol.* 23 (1999) 524.
10 K.S. Patrick, C.D. Kilts, G.R. Breese, *J. Med. Chem.* 24 (1981) 1237.
11. H.C. Kimko, J.T. Cross, D.R. Abernethy, *Clin. Pharmacokinet.* 37 (1999) 457.
12 R.I. Shader, J.S. Harmatz, J.R. Oesterheld, D.X. Parmelee, F.R. Sallee, D.J. Greenblatt, *J. Clin. Pharmacol.* 39 (1999) 775.
13 E. Marchei, M. Farrè, M. Pellegrini, Ó. García-Algar, O. Vall, R. Pacifici, S. Pichini, *Clin. Chem.* 56 (2010) 585.
14 M. La Lande, D.L. Wilson, I.J. VcGilveray, J. *Liq. Chromatogr.* 10 (1987) 2257.
15 G.A. Bach, J. Henion, *J. Chromatogr. B: Biomed. Sci. Appl.* 707 (1998) 275.
16 H.J. Leis, G. Fauler, G. Raspotnig, W. Windischhofer, *J. Mass Spectrom.* 35 (2000) 1100.
17 E. Marchei, M. Farrè, M. Pellegrini, Ó. García-Algar, O. Vall, R. Pacifici, S. Pichini, Forensic *Sci. Int.* 196 (2010) 59.
18 E. Marchei, M. Farrè, M. Pellegrini, S. Rossi, O. García-Algar, O. Vall, S. Pichini, *J. Pharm. Biomed. Anal.*, 49 (2009) 434.
19 P. Jandera, *An. Chim. Acta.* 692 (2011) 1.
20 US Food and Drug Administration, Center for Drug Evaluation and Research, Available: http://www.fda.gov/downloads/Drugs/Guidance/ComplianceRegulatoryInformation/Guidances/ucm070107.pdf (2011).
21 M. Careri, A. Mangia, Anal. *Bioanal. Chem.* 386 (2006) 38.
22 P.J. Taylor, *Clin. Biochem.* 38 (2005) 328.
23 B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, *Anal. Chem.* 75 (2003), 3019.
24 M. Josefsson, I. Rydberg, *J. Pharm. Biomed. Anal.* 55 (2011) 1050.
25 S.M. Paterson, G.A. Moore, C.M. Florkowski, P.M. George, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 881-882 (2012) 20.
26 N.B. Modi, B. Lindemulder, S.K. Gupta, *J. Clin. Pharmacol.* 40 (2000) 379.
27 S. Wang, M. Cyronak, E. Yang, *J. Pharm. Biomed. Anal.* 43 (2007) 701.
28 M. Navarro, S. Pichini, M. Farre, J. Ortuno, P.N. Roset, J. Segura, R. De la Torre, *Clin. Chem*. 47 (2001) 1788.
29 E. Marchei, M. Farré, R. Pardo, O. Garcia-Algar, M. Pellegrini, R. Pacifici, S. Pichini, *Ther. Drug. Monit.* 32 (2010) 508.

TABLE 1 Accuracy, within-day and between day imprecision for the determination of MPH in plasma and saliva (n=6)

	QC sample	Concentration (µg/L)	Accurcay (%)	Within-day imprecision (%)	Between-day imprecision (%)
Plasma	QC1 (LLOQ)	0.5	107.8	5.0	6.9
	QC2	10	101.1	7.0	1.6
	QC3	100	101.3	4.5	1.7
Saliva	QC1 (LLOQ)	0.5	106.2	5.9	5.4
	QC2	10	99.3	2.2	4.2
	QC3	100	98.3	3.4	2.9

FIGURE 1 Molecular structure of methylphenidate (left) and d9-methylphenidate (right).

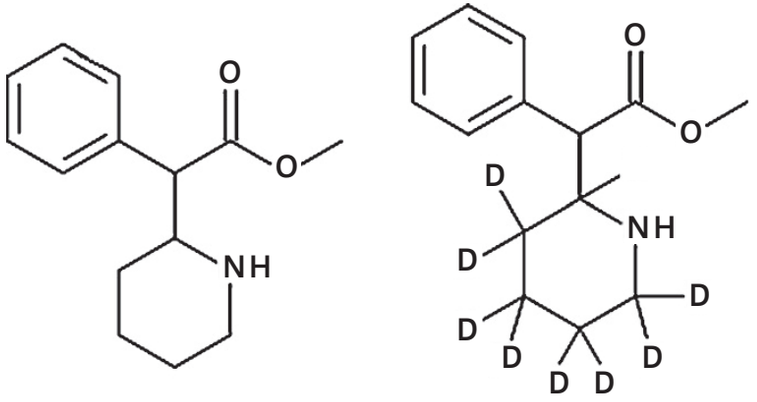


FIGURE 2 Product ion mass (MS2) spectra of $[M+H]^+$ for methylphenidate (top) and $[M+H]^+$ for d9-methylphenidate (bottom).

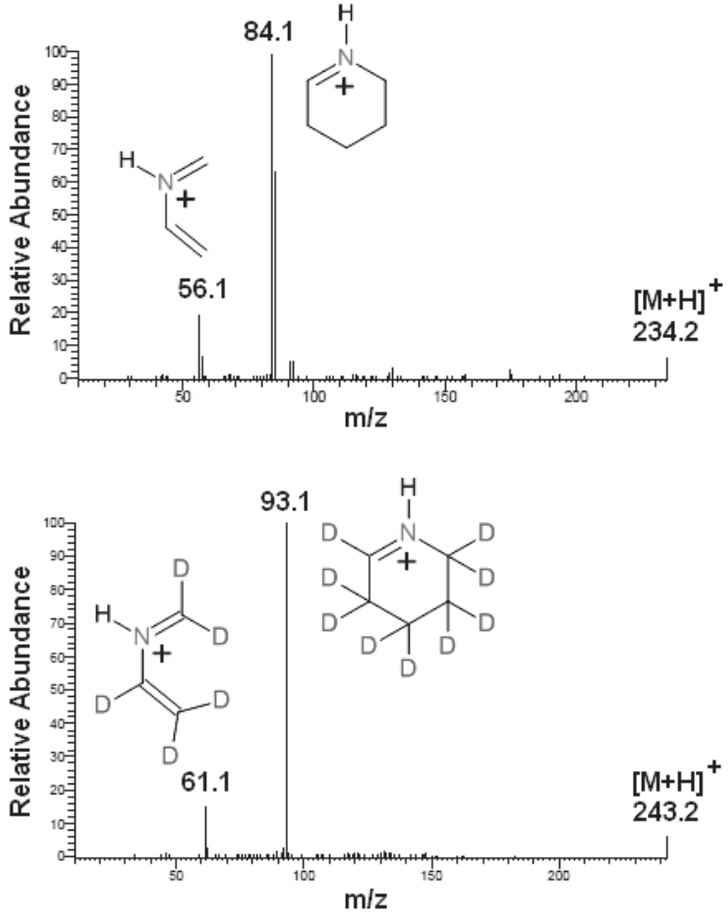


FIGURE 3 **Chromatograms of blanco plasma (A), spiked plasma with a methylphenidate concentration of 5 µg/L (LLOQ) (B) and patient plasma (C). Chromatograms of blanco saliva (D), spiked saliva with a methylphenidate concentration of 0.5 µg/L (LLOQ) (E) and patient saliva (F).**

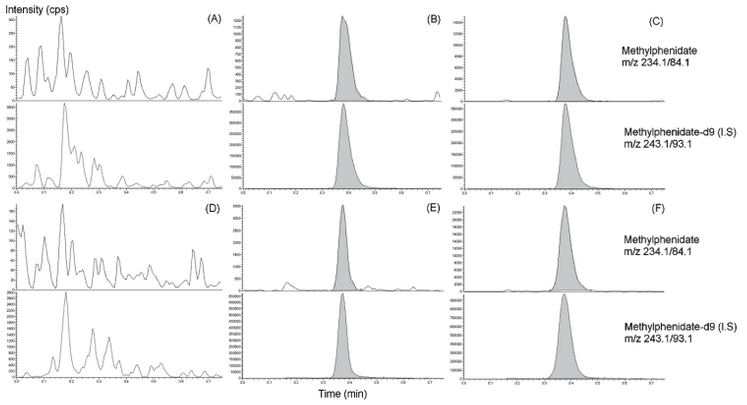


FIGURE 4 **Degradation of methylphenidate in plasma (squares) and saliva (circles) at room temperature.** Closed and open symbols represent the concentration at 0.5 µg/L (QC1 (LLOQ)) and 100 µg/L (QC3), respectively (mean ± SD, n = 3). The fitted lines represent the fitted first-order decay for a concentration of 0.5 µg/L (dashed line) and µg/L (solid line).

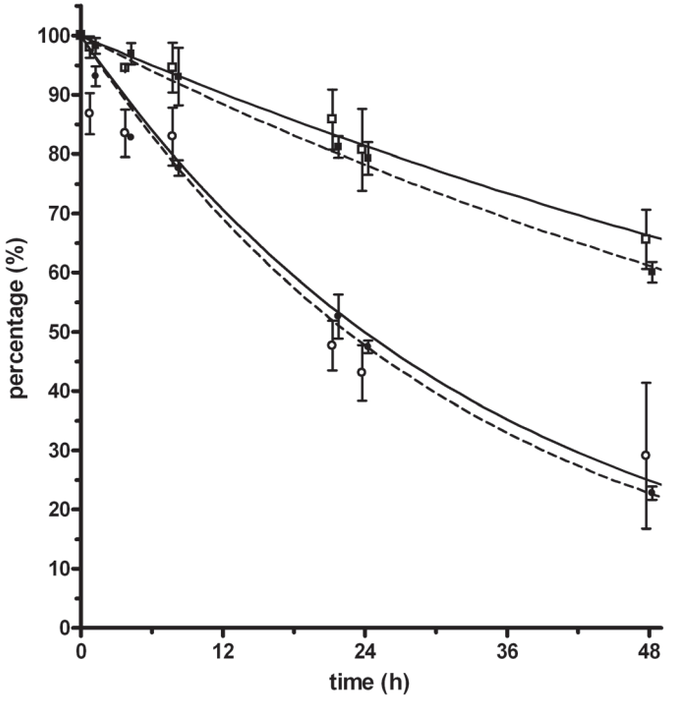
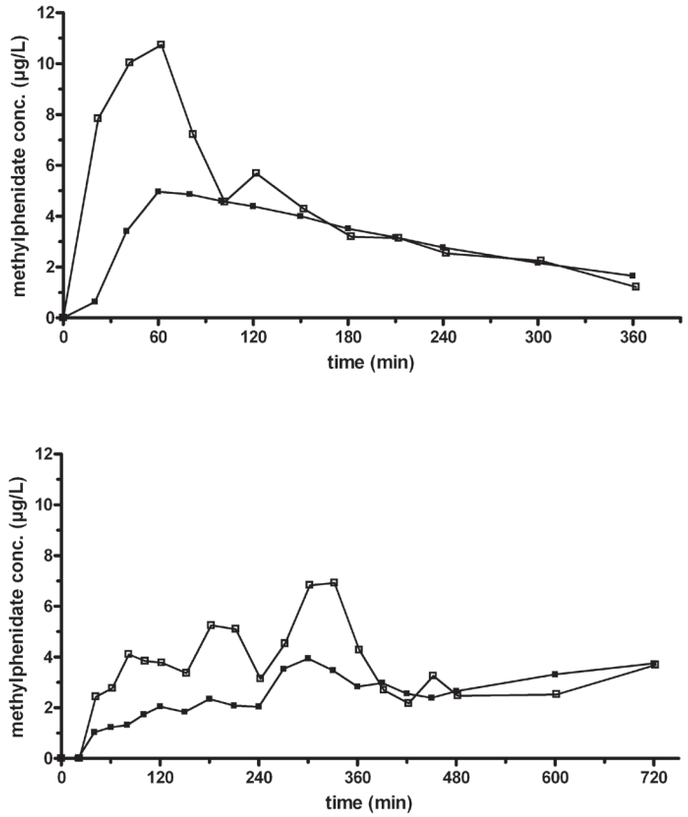


FIGURE 5 **Time profiles of methylphenidate concentration in plasma (closed squares) and saliva (open squares) in a healthy adult volunteer after intake of 10 mg methylphenidate (MPH) in an immediate release formulation (Ritalin; top) and 18 mg MPH in a sustained release preparation (Concerta; bottom) on different occasions.**



CHAPTER 5

Population pharmacokinetics modeling of two methylphenidate formulations in plasma and saliva of healthy subjects

Submitted

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ABSTRACT

Monitoring methylphenidate (MPH) concentrations can help determine whether a lack of observed efficacy and/or the presence of unexpected adverse effects are related to pharmacokinetic (PK) or pharmacodynamic (PD) factors. Saliva sampling is a promising non-invasive alternative to blood sampling, particularly in children. However, the challenges associated with reliably predicting MPH plasma concentration from a saliva sample has limited the feasibility of using saliva sampling to monitor MPH plasma concentration. Here, we investigate and quantify putative sources of variability in MPH plasma and saliva concentrations and describe the saliva-to-plasma relationship using nonlinear mixed-effect population PK modeling. In this randomized, open-label study, immediate-release MPH (MPH-IR) and osmotic release oral system MPH (MPH-OROS) were administered in a crossover design to 12 healthy adult subjects (six men and six women). Paired blood and saliva samples were collected pre-dose and at regular intervals for 6 (MPH-IR) or 11 (MPH-OROS) hours following drug administration. Population PK analysis was performed using nonlinear mixed-effect modeling. A one-compartmental structure model with first-order absorption (with separate compartments for MPH-IR and MPH-OROS) and first-order elimination provided the best description of estimated MPH plasma PK. The estimated clearance was 6.0 liters/hour and the volume of distribution was 7.5 liters. The derived terminal half-life was 0.9 hours. Inter-individual variability was identified on clearance, the volume of distribution, and the absorption rate constant for MPH-OROS. The saliva-to-plasma MPH (S/P) ratio was 2.44 from 2.5 hours onward. Inter-individual variability was identified in the S/P ratio. With proper allometric scaling techniques, we expect that this PK model can used in children to predict the concentration-time profile in the plasma using MPH concentrations measured in saliva samples.

Introduction

Methylphenidate (MPH) is currently the medication of choice for treating patients with attention-deficit/hyperactivity disorder (ADHD), a highly prevalent neurodevelopmental disorder that places significant burdens on social development and can impede academic performance. Although controlled trials have found that 60-70% of children respond to MPH, actual clinical experience has revealed a much lower and less predictable response rate of approximately 50%. The clinical use of MPH is usually based on a trial-and-error approach before optimal therapy is achieved, as MPH has wide inter-individual variability in terms of both plasma concentrations[1,2] and clinical response[3,4]. Approximately 20-30% of patients do not respond favorably to MPH at any dose[5], and these so-called 'non-responders' must switch to an alternative medication after this initial attempt at treatment. Therefore, a significant subset of children with ADHD experience a delay in receiving adequate treatment, and patients may stop taking medication altogether. A clear view of MPH concentration-time profiles is needed in order to understand whether a lack of observed efficacy and/or the presence of unexpected adverse effects is related to pharmacokinetic (PK) or pharmacodynamic (PD) factors. However, measuring circulating MPH concentration in children is extremely challenging due to the need for repeated intravenous blood sampling.

Collecting samples for measuring drug concentrations should be performed with minimal discomfort to the patient, particularly in pediatric patients. Because MPH is a weak base (with a pK_a of 8.9) and has a relatively low molecular weight (233 Da)[6], it diffuses readily across cell membranes and other lipid layers, quickly entering tissues and biological substrates that are more acidic than blood, thus enabling its detection in other matrices at relatively higher concentrations. Moreover, because of its low protein binding saturation (10-33%), nearly all of the total MPH available in the plasma can diffuse to extravascular compartments. Several non-invasive biological matrices for measuring MPH have been proposed, including urine, breath, sweat, hair, and saliva. Saliva sampling is currently the most promising non-invasive alternative to blood sampling, as it allows for the determination of concentration-time profiles of both MPH and the ritalinic acid metabolite. However, several potential complicating factors have been encountered in previous studies, including indications of oral contamination in the first few saliva samples after taking MPH tablets and considerable – yet unexplained – variation in the saliva-to-plasma (S/P) ratio throughout the time course of both tablet and capsule formulations[7]. Nevertheless, if the sources of variability in the S/P ratio could be minimized or quantified, saliva drug sampling has the potential to become a reliable alternative to plasma drug sampling.

Here, our primary objective was to use population-approach modeling techniques to describe the concentration-time profile of MPH in plasma and saliva after oral administration of MPH-IR and MPH-OROS in healthy adult subjects. Our secondary objective was to quantify the degree of contamination in the early saliva samples and to determine the effect of saliva pH on MPH saliva measurements, as MPH's ionized free fraction may be incorporated into saliva as has been described for other weak bases[8-11], including amphetamine-type substances[12,13].

Methods

Clinical trial

This trial was a randomized, open label, two-way crossover study performed in 12 healthy adult subjects (6 men and 6 women). Based on our previous experience, we expected that a sample size of 12 subjects would be sufficient for determining the PK parameters and S/P ratio. The study was conducted in accordance with the International Conference on Harmonisation's Guidelines for Good Clinical Practice and in accordance with the tenets of the Declaration of Helsinki. The study was performed at the Centre for Human Drug Research in Leiden, the Netherlands, and approved by the local ethics committee of Leiden University Medical Center (Leiden, the Netherlands). The subjects

provided written informed consent after receiving a full explanation of the study. Subjects had to be healthy, 18-35 years of age, with a body mass index of 18-30 kg/m^2 and body weight of 50-90 kg. Subjects had to use a medically approved method of contraception throughout the entire study period and for three months after the study was completed.

We excluded subjects with a clinically relevant abnormal history of physical or mental health determined from the subject's medical history and physical examinations (obtained during the screening visit and/or prior to receiving the first dose of the study drug); clinically relevant abnormal laboratory results, ECG, vital signs, or physical findings; current breast-feeding; and/or a history of alcohol and/or substance abuse within three years of screening. Subjects who habitually consumed more than 21 or 14 units of alcohol per week, respectively, and subjects who smoked >5 cigarettes/day or used nicotine or nicotine-containing products within three months of screening were excluded. We also excluded subjects who tested positive for hepatitis B, hepatitis C, or HIV, female subjects with a positive urine-based pregnancy test, and subjects who tested positive for drug and/or alcohol at screening. Subjects with previous exposure to pharmaceutical stimulants (including – but not limited – to MPH, MDMA, methamphetamine, amphetamine, ephedrine, and cocaine) in the past six months were excluded, as were subjects who took any medication other than ibuprofen, paracetamol, oral contraceptives, or topical medication within one week of their first MPH dose. Subjects who participated in an investigational drug study within 90 days of the first dose and/or participated in more than four clinical trials in the past year were not allowed to participate. Finally, subjects who donated blood or lost >500 ml of blood within three months of screening were not allowed to participate.

MPH-IR (immediate release MPH) and MPH-OROS (osmotic controlled-release oral-delivery system MPH) formulations were chosen as interventions, as most Dutch children with ADHD begin their treatment by taking Ritalin (MPH-IR, Novartis Pharmaceuticals UK Ltd.), and Concerta (MPH-OROS, Janssen-Cilag Ltd.) was the most commonly used extended-release formulation at the time of the study (GIP database 2011, *Genees- en hulpmiddelen Informatie).* Each subject randomly received either 10 mg of MPH-IR (Ritalin) or 18 mg MPH-OROS (Concerta) on different study days separated by a minimum of five days. The potential effects of the estrous cycle on MPH pharmacokinetics has not been evaluated in humans[14]. Therefore, female subjects who took oral contraceptives were studied while taking their contraceptive but not in the stop week. Subjects were required to refrain from consuming xanthine- and/or alcohol-containing products and from smoking within 12 hours of MPH administration until the end of each study day. On study days, the subject was questioned regarding his/her intake of medication, alcohol, and/or illegal drugs, and a urine drug screen, a urine-based pregnancy test, and an alcohol breath test were performed before any study-related procedures began. The MPH dose was taken with 240 ml water after an overnight fast of ≥10 hours. Subjects were instructed not to chew the medication and to swallow the tablet or capsule whole. Subjects were confined to the clinical research unit for approximately six (MPH-IR) or 12 (MPH-OROS) hours after drug administration. Water (250 ml) was provided every two hours after the MPH dose to maintain all subjects on a consistent hydration schedule. A standardized lunch (MPH-IR) or lunch and dinner (MPH-OROS) was provided 4 and 10 hours, respectively, after the MPH dose.

To measure the PK of MPH-IR, saliva and blood samples were collected predose and at t=20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, and 360 minutes after the dose. To measure the PK of MPH-OROS, saliva and blood samples were collected at the same time points as well as at the following additional time points: t=270, 330, 360, 390, 420, 450, 480, 600, and 720 minutes. Saliva samples were obtained actively using the Polyester Salivette swab system (Sarstedt AG, Nümbrecht, Germany), a commercially available product designed specifically for collecting saliva specimens. The system contains a roll of polyester that is held in the oral cavity for several minutes. Three swabs were collected per time point. Saliva was collected actively in order to minimize variability in saliva pH. After collection and weighing the saliva (to assess salivary flow), the swabs were immediately centrifuged at 2000x*g* for 10 minutes at 4 °C. Subsequently, the saliva collected from the three swabs was pooled, and pH was measured

using a Symphony pH meter (model SP70P, VWR Scientific) fitted with a pH/Redox electrode (pH range: -2.000 to 19.999; relative accuracy: ± 0.002). Finally, the sample was divided in two, transferred to 2-ml tubes (Sarstedt), and immediately stored at -80 °C. One stored sample was used for analysis, and the other sample was stored as a back-up. Blood samples were collected in 6-ml EDTA tubes to inhibit plasma esterases, which metabolize MPH to ritalinic acid. The blood samples were cooled in an ice bath and centrifuged at 2000x*g* for 10 minutes at 4 °C within 30 minutes of collection. The plasma fractions were collected, aliquoted into two transport tubes (containing approximately 1 ml of plasma per tube) and stored at -80 °C.

Quantification of saliva and plasma MPH *concentration*

MPH (and the internal standard d_9-MPH) was analyzed in plasma and saliva samples by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using the positive ionization mode of a Thermo Scientific (Waltham, MA, USA) Surveyor LC coupled to a Thermo Scientific Quantum Access MS. The development and validation of this LC-MS/MS method using hydrophilic interaction liquid chromatography for MPH analysis and for assessing MPH stability in plasma and saliva at various temperatures have been described previously[15]. The assay's lower limit of quantification (LLOQ) was 0.5 µg/L in both plasma and saliva.

Population model development

DATA

Exploratory individual and summary concentration-time profiles were generated in order to identify potential outliers, to understand the possible effect of censoring concentrations below the LLOQ, and to provide indications regarding the base structural model. All concentrations below the LLOQ after T_{max} were excluded from the analysis. All concentrations below the LLOQ prior to T_{max} were set to zero.

MODELING STRATEGY

Population PK analysis was performed by nonlinear mixed-effect modeling using NONMEM version 7.2.0 (Icon Development Solutions, Ellicott City, MD, USA). The model was developed using ADVAN6 with First-Order Conditional Estimation with Interaction (FOCEI). Different models were compared with increasing complexity in the structural model and by increasing the number of random effects. The objective was to obtain the simplest model that described the data adequately. NONMEM reports an objective function value (OFV), which is the -2 times log likelihood (-2LL). Models were compared using the likelihood ratio test, with the assumption that the difference in -2LL is Chi-square distributed, with degrees of freedom determined by the number of additional parameters in the more complex model. Hence, with a decrease in OFV of at least -6.63 points ($p<0.01$), the model with one additional parameter was preferred over its parent model. We also used graphical analyses to help assess the differences between models. These analyses included: (1) predicted concentration versus observed concentration; (2) Conditional Weighted Residuals with Interaction (CWRESI) versus predicted concentration, as well as CWRESI versus time; (3) frequency distribution of the CWRESI; (4) frequency distributions of the post hoc individual estimates of ETAs; and (5) correlation plots of post hoc individual estimates of ETAs of all parameters with a random effect. The statistics software package R was used for graphical representations to evaluate of the goodness of fits, to select covariates, and to evaluate the models.

POPULATION PK MODEL DEVELOPMENT

The population PK analysis focused on identifying structural (e.g., 2 and 3 compartmental) models with appropriate absorption and elimination processes (e.g., linear or nonlinear) to best describe and explain all of the collected data. The population parameter estimates were incorporated using ln-normal distributions. Additional, proportional or combined additive and proportional residual error distributions were drawn using parameters from a normal distribution to describe the residual variability. The random effects structure was incorporated using ln-normal distributions for the inter-individual variability

(IIV) of the PK parameters. The IIV of the PK parameters were established by applying an exponential transformation of a normal random effects distribution. Various types of variance-covariance matrices were tested for IIV. The estimated population values (both fixed and random effects) were used to determine individual empirical Bayes' estimates (post hoc estimates) of the PK parameters. The best structural PK model was determined before any covariate (e.g., sex, weight, or height) was evaluated for incorporation into the model. To visualize potential correlations, scatter plots were created for each pair of covariates with a variance-covariance ellipse, the Pearson correlation coefficient, and its significance. This approach was performed to assess correlations between covariates, correlations between post hoc individual estimates of ETA for each parameter and the covariate, and between estimated PK parameters and covariates. Confounding covariates were grouped following an evaluation of their correlation structure. The covariate in each variable cluster that had the highest correlation with the empirical Bayes' estimates of the parameters – and which was also clinically relevant – was implemented in the model. Continuous covariates were included by centering on a reference value; the median of the observed covariate values was selected as the most informative reference value.

The model was developed using a sequential approach in which plasma MPH PK was modeled first. Subsequently, a saliva MPH PK model was developed in which all plasma PK parameters were fixed to the individual estimates derived from the plasma PK model.

Results

Subjects

Twelve healthy adult subjects (6 men and 6 women) met the selection criterion and were enrolled in the study. The mean age of the subject was 23 years (range: 19-31 years), and the subjects had a mean body mass index of 22.1 kg/m^2 (range: 19.7–26.6 kg/m^2). All subjects tested negative for pre-dose drugs of abuse in the urine. Concomitant medication used during the study period included ibuprofen (400 mg orally four days prior to the study day) and paracetamol (500 mg orally eight days after the last dose). All 12 subjects completed the study.

Pharmacometrics analysis

POPULATION PK PLASMA MODEL DEVELOPMENT

A total of 764 plasma samples were obtained from the 12 subjects. Fewer than 20% of the samples had PK data below LOQ. Based on the exploratory plots, one outlier (a plasma sample taken five hours after the administration of an MPH-IR dose) was excluded from the analysis. After the administration of MPH-IR and MPH-OROS, the concentration-time profiles of subject 8 and subject 9 deviated from the profiles of the other subjects; however these data remained in the dataset.

DEVELOPMENT OF THE POPULATION PK PLASMA MODEL

The plasma PK data were described best by a one-compartment PK model with separate absorption compartments for the two drug formulations. The best model is depicted schematically in Figure 1. We estimated the lag time between administration of the drug and the onset of absorption (ALAG). For the MPH-OROS formulation, absorption was divided into two first-order absorption processes; one standard oral absorption process reflecting the capsule's immediate-release component, with its own lag time and the same ka_1 as the IR formulation ($OROS_{IR}$), and an additional absorption process reflecting the capsule's slow-release component ($OROS_{SR}$), which was defined as a continuous infusion with a distinct lag time and ka_2. This approach required the estimation of an infusion rate (R3). To parse the MPH-OROS dose into the $OROS_{IR}$ and $OROS_{SR}$ components, the fraction of the dose corresponding to $OROS_{IR}$ was modeled in terms of bioavailability (F), and the remaining fraction of the dose (corresponding to $OROS_{SR}$) was defined as (1-F). The central compartment was

defined in terms of distribution volume (V) and clearance (CL). Ultimately, we abandoned our attempts to model IR and $OROS_{IR}$ as one compartment, as this resulted in worse fit and a non-normal distribution of the individual CL parameters (in which CL was formulation-dependent) and conditional weighted residuals. IIV was identified for CL, V, and ka_2. Based on the correlation scatter plots, age and sex were considered as covariates for CL, V, and ka_2; these were not incorporated in the model as they did not result in an improvement in OFV. Covariance between V and ka_2 improved the model's performance and was therefore kept in the model. The estimated PK parameters of the best PK plasma model are summarized in Table 1. Parameter estimations are accurate given the relatively low standard deviations, except for the absorption rate constants that show higher, but acceptable standard deviations. With regard to the goodness-of-fit plots (Figure 2), the observations versus population predictions indicate that the structural model is appropriate, as inclusion of the IIV (individual predictions) improves the goodness-of-fit, e.g. the observations are closer to the line of unity. In general, the conditional weighted residuals versus the population prediction and versus time are symmetrically distributed around zero indicating good model performance. However, there is a small –albeit acceptable– bias in the low concentration range near time=0. Overall, the conditional weighted residuals are normally distributed (Figure 2). The individual plasma MPH concentration versus time after administration of MPH-IR and MPH-OROS are well described by the model, with exception for subjects 8 and 9 (Figure 3).

DEVELOPMENT OF THE POPULATION PK SALIVA MODEL

A total of 612 saliva samples were obtained from the 12 subjects. Fewer than 20% of the samples had PK data that was below LOQ. The saliva samples collected after administration of MPH-IR (tablet formulation) had clear indications of contamination, and efforts to correct for this contamination resulted in major bias in the description of the terminal PK phase. Based on previous experience in similar studies (our unpublished data), we excluded all post-dose MPH-IR data collected prior to the 2.5-hour post-dose time point.

The saliva PK data were described best as a linear relationship between MPH concentration in the plasma and MPH concentration in the saliva. The individual plasma MPH drug concentrations at each time point were used to drive the saliva model, with the following equation:

$$Y = \alpha \times C_p$$

where Y is the saliva MPH concentration, C_p is the plasma MPH concentration, and α is the estimated parameter (S/P ratio).

The estimated S/P ratio (α) was 2.44 (± standard error 26.9%). The residual error was described best by a proportional error structure (0.171 ± standard error 1.3%). IIV could be identified for α (0.14 ± standard error: 2.9%). No covariate relationships could be identified (for example, saliva pH or flow were not identified as covariates).

As with the best PK plasma model, the predicted concentrations in the best PK saliva model were accurate with a small – albeit acceptable – bias in the low concentration range (Figure 4). The first samples also had a time-dependent bias (Figure 4). CWRESI was distributed normally, with 0 lying within the 1.5 interquartile range, despite the presence of some outliers at the extremes of the distribution (Figure 5); these outliers remained when the data were separated by formulation (data not shown). The outliers at the extremes were more evident for MPH-OROS than for MPH-IR. With the exception of the data collected from subjects 8 and 9 following MPH-IR administration, the model describes the data adequately (Figure 6).

Discussion

Reliabe prediction of plasma MPH concentrations based on saliva sampling is challenging. As a result, the feasibility of this method has been questioned[16]. Minimizing and quantifying sources of variability in the saliva/plasma (S/P) ratio could improve the acceptance of serial saliva sampling as an alternative to therapeutic drug monitoring using (invasive) serial plasma sampling, which

is of particular interest in pediatric populations. Here, a first attempt was made to quantify sources of variability in MPH plasma and saliva concentrations, and to describe the relationship between MPH concentration in saliva and MPH concentration in plasma using a population PK modeling approach. The data were comprised of paired plasma and saliva MPH concentrations from healthy adults, following a single dose of MPH-IR (through 6 hours post-dose) or MPH-OROS (through 24 hours post-dose).

A one-compartment model with first-order absorption (separate absorption compartments for MPH-IR and MPH-OROS) and first-order elimination best described the plasma PK for MPH. The population parameter estimates for clearance was 403 liters/hour with a distribution volume of 1808 liters and a derived terminal half-life (ln2/ke) of 3.15 hours, which is consistent with previously published estimates[17,18]. The population parameter estimates for clearance, distribution volume, and lag time (for the IR formulation) had low uncertainty; however, the standard errors of the population values for the absorption rate constants of both MPH-IR and MPH-OROS, as well as the standard error for the lag time of the sustained release phase of MPH-OROS, were relatively high, albeit still within an acceptable range. Absolute bioavailability after oral dosing has been reported to be both low and variable[19]. The level of uncertainty for ka's and lag time may be explained – at least in part – by the relatively low number of subjects, lack of data points in the upward part of the concentration time profile and the seemingly aberrant PK profiles of two subjects, particularly after receiving the MPH-IR formulation. For example, the post-MPH-IR dose plasma concentrations in subject 9 had an extremely long absorption phase, which may be attributed to extended gastric emptying time, which is the primary factor controlling MPH absorption for IR formulations[20]. Because gastric emptying time can be prolonged in both clinical and research settings, we did not exclude these data from our analysis. In addition, large differences between subjects have been reported with respect to the release profile of MPH-OROS[21]. Finally, our model described absorption during the osmotic release phase of MPH-OROS capsules as a continuous, stable infusion. In contrast, the rate of release rate from OROS capsules has been reported to increase over time due to the drug's concentration gradient that is incorporated into the two layers[22]. Therefore, the model's descriptive properties might be improved further by incorporating previously published data – for example, the average release profile of MPH-OROS capsules – into the model.

Given MPH's physicochemical characteristics, it is likely that MPH is incorporated into the saliva by passive diffusion of the free ionized drug fraction, which will become ionized in saliva and therefore cannot diffuse back into the plasma[23]. A small – but acceptable – bias in the low concentration range was observed in saliva, which was likely due to the similar bias in the plasma PK model and oral contamination in the saliva samples collected early after MPH-IR administration. Our data set included some subjects who had contamination in their early samples (i.e., some or all of the subjects after taking MPH-IR) and subjects who had no contamination (i.e., all of the subjects after taking MPH-OROS); by inference information collected after taking MPH-OROS could theoretically provide information regarding possible contamination after taking MPH-IR. Such contamination would be the strongest – and therefore would have the highest impact on PK parameters – at earlier time points. Our efforts to correct for oral contamination resulted in a large bias in the description of the terminal phase. Therefore, a pragmatic approach was chosen: all saliva data collected in the first 2.5 hours after administration were excluded from analysis.

Obtaining an accurate S/P ratio is essential for realizing the full potential of using saliva sampling to monitor plasma MPH concentrations. The theoretical S/P ratio based on the modified Henderson-Hasselbalch equation has previously been calculated as 3.1[24]. In our study, the model-based S/P ratio for MPH at time points beyond 2.5 hours after administration averaged 2.44. This value is lower than the average S/P ratios reported in previous studies[7,25], which may be explained by study-related differences in saliva stimulation. Because stimulating saliva secretion increases the saliva's pH to values that approach plasma pH, the apparent drug concentration of basic drugs can be reduced, and resulting S/P ratios will have less variability, for example as described previously for MDMA and cocaine[26,27]. Therefore, we obtained the saliva samples

actively using a mechanical stimulus (which can stimulate saliva flow of 1-3 ml/min[28]). In contrast, in a previous study[12] samples were collected by having the subject spit, which usually produces little stimulation, thus leading to higher apparent drug concentrations and higher S/P ratio variability. As basal (i.e., unstimulated) salivary flow is generally lower in children with ADHD than in children without ADHD[29], stimulating salivary flow with active sampling may be even more important in this patient population in order to ensure adequate sample volume for analysis. The S/P ratios in our study had inter-individual variability. For strong basic drugs such as methamphetamine, the S/P ratio can be highly sensitive to small changes in saliva pH, and inter-individual variability in saliva pH is the likely explanation for inconsistent S/P ratios with these drugs types[30]. However, both saliva pH and saliva flow could not be identified as covariates in the current dataset.

Our results serve as the impetus for exploring further the feasibility using saliva as a non-invasive method for monitoring MPH concentrations in patients, particularly children. Because this method has the added benefit of allowing on-site testing without the need for medical personnel or complicated sample processing, the burden of collecting samples from children is decreased even further. Using saliva sampling to measure MPH concentration is currently limited to confirming treatment compliance or testing for treatment misuse. However, monitoring MPH saliva concentrations in children with ADHD could have several important clinical and research applications. For example, the time course of clinical efficacy parameters in children can be simulated based on the time course of MPH concentration in adults[31]. If our model is validated for children using pediatric PK data, the true parameters of the pediatric PK-PD relationship could be estimated, and the model could be used to estimate target MPH concentrations > 2.5 hours after administration in the pediatric population. Such a result would represent an important step towards using non-invasive therapeutic drug monitoring to provide customized treatment to children with ADHD. Ultimately, the ability to differentiate between responders and non-responders – ideally at the onset of MPH treatment – would help clinicians determine which medication, dose, and formulation will likely work best in each child with ADHD. In addition, our PK model could be used to simulate a clinical trial in order to determine the optimum sampling schedule for future studies.

Despite its advantages, several issues may limit the potential applicability of our method. In our study, concomitant medication shortly before and/or during study days was limited to the use of only a few medications, including ibuprofen and paracetamol. Children with ADHD often use medications that are related – either directly or indirectly – to ADHD treatment or the treatment of psychiatric comorbidities; such medication include tricyclic antidepressants and other antidepressants, clonidine, antipsychotics, antiepileptic agents, anxiolytics, melatonin, and other hypnotics[32]. Thus, validation studies are needed to in order to determine the predictive performance of our model in patients who use these medication types.

In conclusion, we report that the relationship between plasma and saliva MPH concentrations in healthy adult subjects can be described as a constant S/P ratio, but only 2.5 hours after administration of two distinct oral formulations of MPH. With proper allometric scaling (using body size to account for developmental changes in MPH clearance and distribution volume), this PK model may also be suitable for predicting the concentration-time plasma MPH profile in children using non-invasive saliva sampling. Further studies are needed to determine the predictive performance of the model in children with ADHD.

REFERENCES

1 Shaywitz SE, Hunt RD, Jatlow P, Cohen DJ, Young JG, Pierce RN, et al. Psychopharmacology of attention deficit disorder: pharmacokinetic, neuroendocrine, and behavioral measures following acute and chronic treatment with methylphenidate. *Pediatrics.* 1982;69:688-694.

2 Gualtieri CT, Hicks RE, Patrick K, Schroeder SR, Breese GR. Clinical correlates of methylphenidate blood levels. *Ther Drug Monit.* 1984;6:379-392.

3 Rapport MD, Denney C, DuPaul GJ, Gardner MJ. Attention deficit disorder and methylphenidate: normalization rates, clinical effectiveness, and response prediction in 76 children. *J Am Acad Child Adolesc Psychiatry.* 1994;33:882-893.

4 Rapport MD, Kelly KL. Psychostimulant effects on learning and cognitive function: findings and implications for children with attention deficit hyperactivity disorder. *Clin Psychol Rev.* 1991;11:61-92.

5 Markowitz JS, Straughn AB, Patrick KS. Advances in the pharmacotherapy of attention-deficit-hyperactivity disorder: focus on methylphenidate formulations. *Pharmacotherapy.* 2003;23:1281-1299.

6 Methylphenidate, ChemSpider ID (CSID): 4015. ChemSpider . 2013. 11-4-2013.

7 Marchei E, Farre M, Pardo R, Garcia-Algar O, Pellegrini M, Pacifici R, et al. Correlation between methylphenidate and ritalinic acid concentrations in oral fluid and plasma. *Clin Chem.* 2010;56:585-592.

8 Kidwell DA, Holland JC, Athanaselis S. Testing for drugs of abuse in saliva and sweat. *J Chromatogr B Biomed Sci Appl.* 1998;713:111-135.

9 Cone EJ. Saliva testing for drugs of abuse. *Ann N Y Acad Sci.* 1993;694:91-127.

10 Jenkins AJ, Oyler JM, Cone EJ. Comparison of heroin and cocaine concentrations in saliva with concentrations in blood and plasma. *J Anal Toxicol.* 1995;19:359-374.

11 Moolchan ET, Cone EJ, Wstadik A, Huestis MA, Preston KL. Cocaine and metabolite elimination patterns in chronic cocaine users during cessation: plasma and saliva analysis. *J Anal Toxicol.* 2000;24:458-466.

12 de la Torre R, Farre M, Navarro M, Pacifici R, Zuccaro P, Pichini S. Clinical pharmacokinetics of amfetamine and related substances: monitoring in conventional and non-conventional matrices. *Clin Pharmacokinet.* 2004;43:157-185.

13 Navarro M, Pichini S, Farre M, Ortuno J, Roset PN, Segura J, et al. Usefulness of saliva for measurement of 3,4-methylenedioxymethamphetamine and its metabolites: correlation with plasma drug concentrations and effect of salivary pH. *Clin Chem.* 2001;47:1788-1795.

14 Patrick KS, Gonzalez MA, Straughn AB, Markowitz JS. New methylphenidate formulations for the treatment of attention-deficit/hyperactivity disorder. *Expert Opin Drug Deliv.* 2005;2:121-143.

15 Secilir A, Schrier L, Bijleveld YA, Toersche JH, Jorjani S, Burggraaf J, et al. Determination of methylphenidate in plasma and saliva by liquid chromatography/tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2013;923-924:22-28.

16 Papaseit E, Garcia-Algar O, Simo S, Pichini S, Farre M. [Methylphenidate in the treatment of children with attention-deficit hyperactivity disorder: monitoring in biological matrices]. *An Pediatr (Barc).* 2013;78:123-10.

17 Aoyama T, Kotaki H, Sasaki T, Sawada Y, Honda Y, Iga T. Nonlinear kinetics of threo-methylphenidate enantiomers in a patient with narcolepsy and in healthy volunteers. *Eur J Clin Pharmacol.* 1993;44:79-84.

18 Markowitz JS, Straughn AB, Patrick KS, DeVane CL, Pestreich L, Lee J, et al. Pharmacokinetics of methylphenidate after oral administration of two modified-release formulations in healthy adults. *Clin Pharmacokinet.* 2003;42:393-401.

19 Chan YM, Soldin SJ, Swanson JM, Deber CM, Thiessen JJ, Macleod S. Gas chromatographic/mass spectrometric analysis of methylphenidate (ritalin) in serum. *Clin Biochem.* 1980;13:266-272.

20 Patrick KS, Gonzalez MA, Straughn AB, Markowitz JS. New methylphenidate formulations for the treatment of attention-deficit/hyperactivity disorder. *Expert Opin Drug Deliv.* 2005;2:121-143.

21 Markowitz JS, Straughn AB, Patrick KS, DeVane CL, Pestreich L, Lee J, et al. Pharmacokinetics of methylphenidate after oral administration of two modified-release formulations in healthy adults. *Clin Pharmacokinet.* 2003;42:393-401.

22 Summary Product Characteristics Concerta. 2014.

23 Marchei E, Farre M, Pardo R, Garcia-Algar O, Pellegrini M, Pacifici R, et al. Correlation between methylphenidate and ritalinic acid concentrations in oral fluid and plasma. *Clin Chem.* 2010;56:585-592.

24 Kidwell DA, Holland JC, Athanaselis S. Testing for drugs of abuse in saliva and sweat. *J Chromatogr B Biomed Sci Appl.* 1998;713:111-135.

25 Josefsson M, Rydberg I. Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry--a method applied on clinical and forensic investigations. *J Pharm Biomed Anal.* 2011;55:1050-1059.

26 Gorodischer R, Koren G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. *Dev Pharmacol Ther.* 1992;19:161-177.

27 Kato K, Hillsgrove M, Weinhold L, Gorelick DA, Darwin WD, Cone EJ. Cocaine and metabolite excretion in saliva under stimulated and nonstimulated conditions. *J Anal Toxicol.* 1993;17:338-341.

28 Kato K, Hillsgrove M, Weinhold L, Gorelick DA, Darwin WD, Cone EJ. Cocaine and metabolite excretion in saliva under stimulated and nonstimulated conditions. *J Anal Toxicol.* 1993;17:338-341.

29 Hidas A, Noy AF, Birman N, Shapira J, Matot I, Steinberg D, et al. Oral health status, salivary flow rate and salivary quality in children, adolescents and young adults with ADHD. *Arch Oral Biol.* 2011;56:1137-1141.

30 Mucklow JC, Bending MR, Kahn GC, Dollery CT. Drug concentration in saliva. *Clin Pharmacol Ther.* 1978;24:563-570.

31 Kimko H, Gibiansky E, Gibiansky L, Starr HL, Berwaerts J, Massarella J, et al. Population pharmacodynamic modeling of various extended-release formulations of methylphenidate in children with attention deficit hyperactivity disorder via meta-analysis. *J Pharmacokinet Pharmacodyn.* 2012;39:161-176.

32 Hodgkins P, Sasane R, Meijer WM. Pharmacologic treatment of attention-deficit/hyperactivity disorder in children: incidence, prevalence, and treatment patterns in the Netherlands. *Clin Ther.* 2011;33:188-203.

TABLE 1 Parameter estimates of the best plasma MPH PK model.

Parameter	Estimate* (standard error)	IIV (standard error)
CL (L/h)	5.951 (8.0)	0.065 (3.7)
V (L)	7.484 (9.8)	0.043 (2.3)
ka_1 (h^{-1})	1.075 (26.3)	
$ALAG_1$ (h)	-1.208 (2.4)	
R_3 (mg/h)	15.184 (8.8)	
F_2 (*100%)	0.272 (2.6)	
$ALAG_2$ (h)	-1.338 (12.3)	
$ALAG_3$ (h)	0.487 (19.1)	
ka_2 (h^{-1})	-1.081 (23.0)	0.244 (20.4)
Covariance	0.0851 (7.1)	
Proportional error	0.1 (0.99)	

IIV, inter-individual variability.

FIGURE 1 Schematic representation of best PK plasma model of MPH-IR and MPH-OROS. ALAG, lag time between administration and onset of absorption; central, central compartment; ka, absorption rate constant; ke, elimination rate constant; IR, absorption compartment of immediate release methylphenidate; $OROS_{IR}$, absorption compartment of immediate release part of osmotic controlled-release oral-delivery system methylphenidate; $OROS_{SR}$, absorption compartment of sustained release part of osmotic controlled-release oral-delivery system methylphenidate.

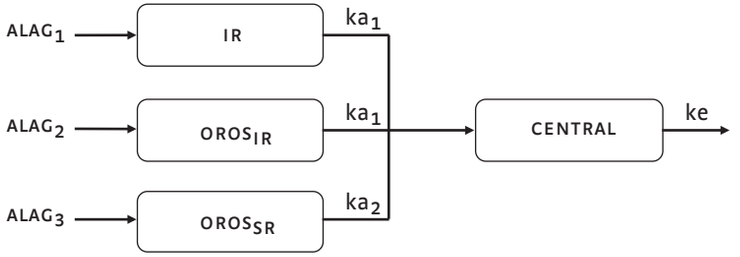


FIGURE 2 Goodness of fit plots of the plasma PK model. Upper left: observed (DV) versus population predicted (PRED) plasma MPH concentrations (black line is the line of unity); upper right: DV versus individual predicted concentrations (IPRE (black line is the line of unity); lower left: conditional weighted residuals (CWRESI) versus PRED; lower right: CWRESI versus time (TIME).

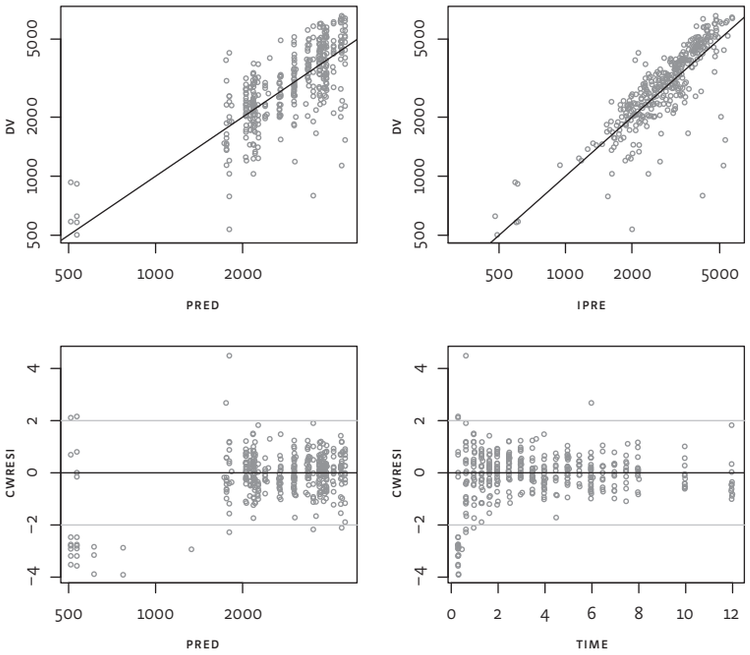
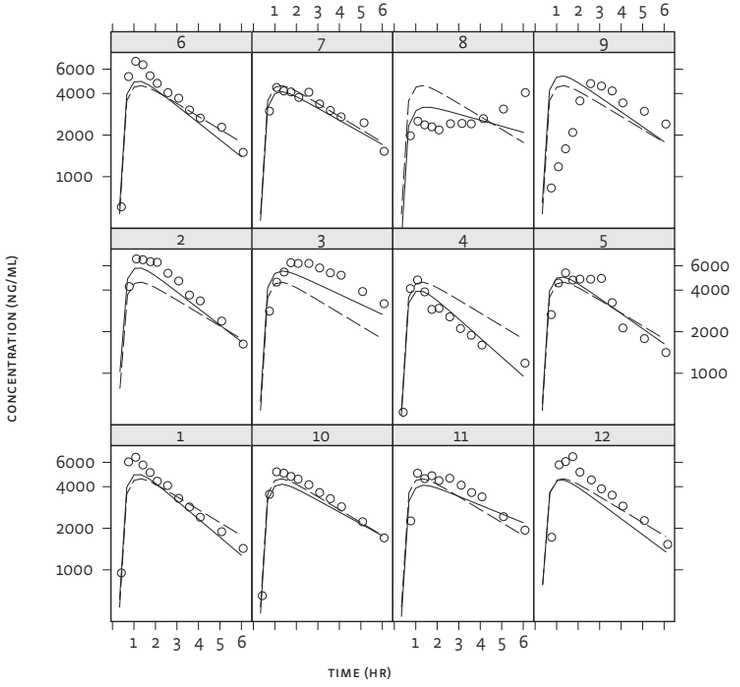


FIGURE 3 Individual plasma MPH concentration versus time after administration of MPH-IR (above) or MPH-OROS (under), plotted on a log-linear scale. Dashed lines represent the population prediction, continuous lines represent the individual prediction, and circles represent the observations.



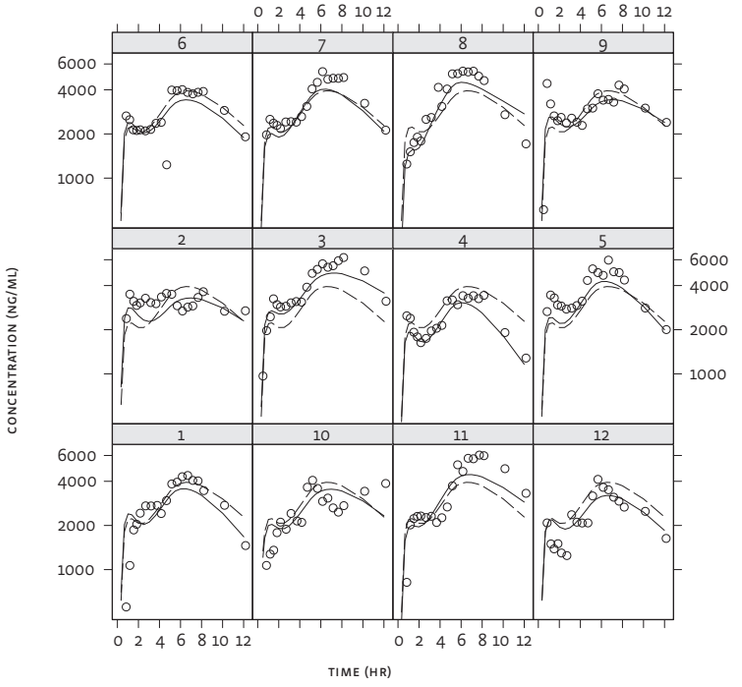


FIGURE 4 **Goodness of fit plots of the saliva PK model.** Upper left: observed (DV) versus population predicted (PRED) plasma MPH concentrations (black line is the line of unity); upper right: DV versus individual predicted concentrations (IPRE (black line is the line of unity); lower left: conditional weighted residuals (CWRESI) versus PRED; lower right: CWRESI versus time (TIME).

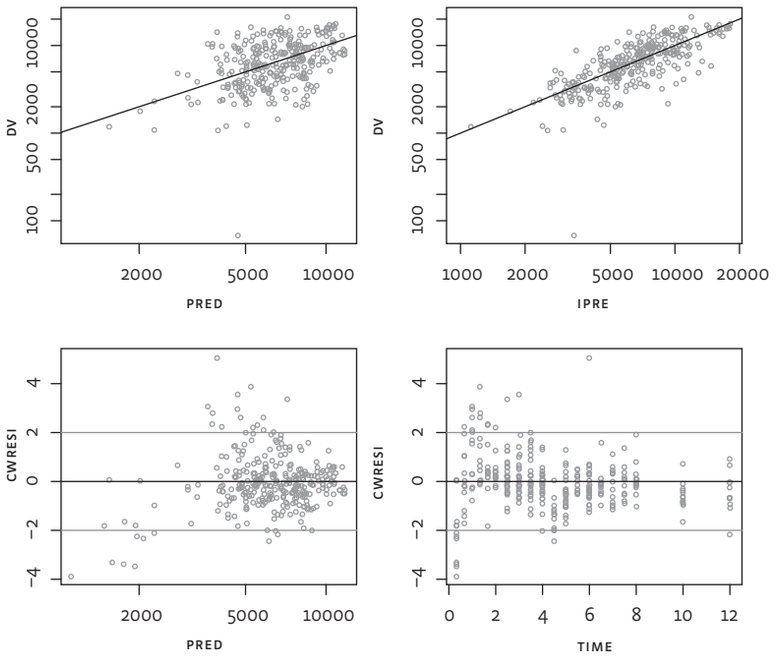


FIGURE 5 **Pooled distribution of MPH-IR and MPH-OROS CWRESI results**, visualized as a frequency histogram (left; the line represents a normal distribution), a box plot (middle), and a QQ plot (right).

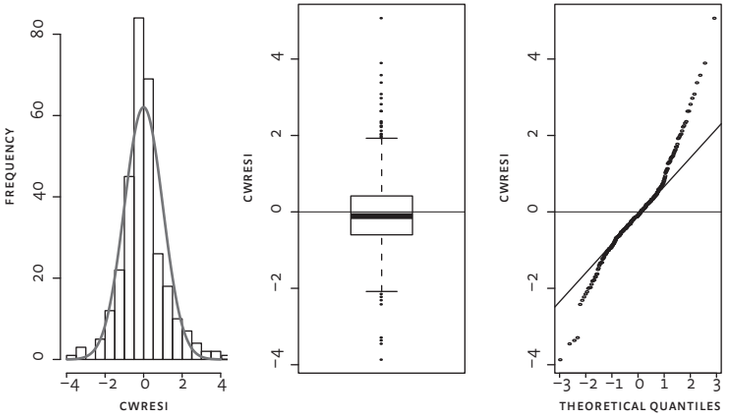
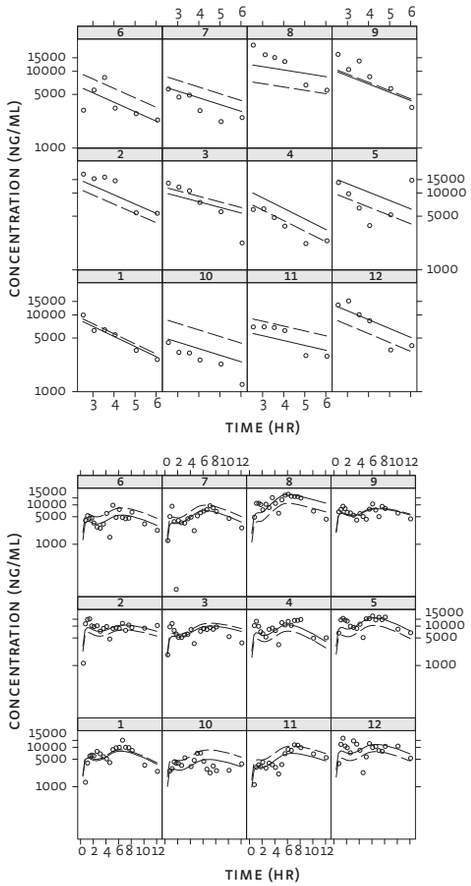


FIGURE 6 Individual saliva MPH concentration versus time after administration of MPH-IR (above) or MPH-OROS (under), plotted on a log-linear scale. Dashed lines represent the population prediction, continuous lines represent the individual prediction, and circles represent the observations.



CHAPTER 6

Caffeine pharmacokinetics and effects on central and autonomous nervous system parameters in adolescents

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ABSTRACT

Despite the highly prevalent use of caffeine among adolescents, remarkably little research has been conducted regarding the physiological and behavioral effects of caffeine in this age group. Data obtained from animal studies suggest that the effects of caffeine reported in adults cannot be extrapolated simply to adolescents. Therefore, we evaluated the effect profile of caffeine on central and autonomic nervous system parameters following the consumption of a low dose caffeinated beverage by healthy adolescents; the results were compared with data obtained following the consumption of a non-caffeinated beverage. Caffeine concentrations were measured from saliva samples. In a separate study using adult volunteers, we determined the extent of oral contamination with caffeine after consuming a caffeinated beverage versus swallowing a caffeine capsule. In addition, because previous studies in adults correlated caffeine's effects with changes in plasma concentration (but not saliva concentration), both saliva and plasma samples were collected simultaneously in order to measure the saliva-to- plasma (S/P) ratio of caffeine concentration. Based on the data collected from this kinetic study, a population PK model was built to estimate plasma drug levels in adolescents; this model could prove useful to develop a PK/PD model. In adolescents, caffeine had significant effects on task parameters related to attention and visuomotor coordination (adaptive tracking task) and alertness (saccadic peak velocity). The cognitive effects of caffeine included an increase in error rate in the attention switch task. Plasma caffeine concentrations in adults were described best as a two-compartment model with a dose depot, first-order absorption kinetics, and first-order elimination kinetics. Lean body mass-dependent variability was identified for the volume of the central compartment. This PK model was expanded to a population model that described saliva caffeine concentrations in adults >1 hour after administration as a fraction (0.68) of plasma concentration. Caffeine's early effects in adolescents (i.e., within one hour) were not suitable for inclusion in a PK/PD model. In conclusion, in healthy, alert adolescents, low-dose caffeine has significant effects on parameters regarding alertness and reaction speed. Whether these effects observed in adolescents are larger in adolescents than in adults remains to be determined.

Introduction

Caffeine (1,3,7-trimethylxanthine) is the most commonly used psychoactive substance worldwide[1]; caffeine is widely available in foods, dietary supplements, chewing gum, beverages, and many over-the-counter combination analgesics. Many adolescents use caffeine as a way to enhance their academic or athletic performance[2] and to intentionally postpone sleep during nighttime leisure activities[3]. Given that some caffeine-containing products are marketed directly to children and adolescents, and given that caffeine use among children and adolescents has increased by 70% since 1977[4], it is important to understand better the potential effects – both positive and negative – of caffeine use within this particular population[2]. An extensive body of research regarding the behavioral and psychomotor effects of caffeine in adults shows that caffeine produces behavioral effects that are similar to the effects of 'classic' central nervous system (CNS) stimulants such as cocaine and amphetamine. However, despite its high incidence of use among adolescents, remarkably little research has been conducted regarding the physiological and behavioral effects of caffeine in this specific age group[2,5].

Caffeine's effects in adolescents can differ from its effect in adults[2]. To date, no studies have compared the effects of caffeine between adolescents and adults. However, prepubescent children experience more objective effects of caffeine than adults at doses of 3-10 mg/kg; these effects include increased motor activity, increased speech rate, and decreased reaction time. After ingesting caffeine, adults generally report side effects that children do not appear to experience; however, autonomic measures of arousal are affected similarly in both age groups[6]. A relatively limited number of animal studies regarding the correlation between age and caffeine's effects have shown that caffeine-induced locomotor stimulation is higher in adolescent rats than in adult rats[7], and adolescent rats may respond more robustly than adult rats to adaptive changes associated with chronic caffeine consumption[8]. These age-dependent effects are likely not limited to caffeine, as age-dependent effects have been reported for other CNS stimulants – including amphetamine[9], cocaine[10,11], and methylphenidate[12,13] – potentially due to the functional inhibition of dopamine D_1 receptors at young age[11]. Based on these findings, the effects of caffeine that have been reported in adults likely cannot be extrapolated simply to adolescents.

Here, we measured the effect profile of caffeine on both central and autonomic nervous system parameters after consuming two cups of espresso by adolescents who usually infrequently consume caffeine. Saliva samples were collected in order to measure caffeine concentration. Because the saliva can be contaminated by residual caffeine after drinking a caffeinated beverage, we performed a second study in young adult volunteers to compare the extent of contamination between drinking a caffeinated beverage and swallowing a caffeine capsule. In addition, although caffeine's effects have been correlated to changes in plasma caffeine concentrations[14,15], the relationship between caffeine's effect and saliva concentrations has not been studied. Therefore, we collected saliva and plasma samples simultaneously in order to measure the saliva-to-plasma (S/P) ratio for caffeine concentration. Based on the data obtained from this kinetic study, a population pharmacokinetic (PK) model was developed to estimate plasma drug levels in adolescents, and this model could potentially be used to develop a PK/PD model.

Methods

Subjects

ADOLESCENT STUDY

Healthy male and female adolescents (15-18 years of age) were included. The subjects were non-smokers and consumed ≤14 units of alcohol per week. After providing written informed consent (plus consent from a parent or legal guardian for subjects <18 years of age), the subjects were medically screened within three weeks of the start of the study; subjects who presented with relevant clinical abnormalities were excluded. We also excluded subjects who used

medications and/or agents known to affect caffeine metabolism and/or CNS performance; urine drug screens were performed during the selection process and prior to each test day. The study was approved by the Central Committee on Research involving Human Subjects.

KINETIC ADULT STUDY

The inclusion criteria for this study included healthy male or female volunteers 18-35 years of age with a body mass index of 18-30 kg/m^2 and body weight of 50-90 kg. Exclusion criteria included a personal history of impaired physical or mental health; a history of drug, substance, and/or alcohol abuse; and abnormal findings with respect to medical history, physical examination, ECG, vital signs, and/or blood and urine laboratory results. The subjects were instructed not to use any medications, dietary supplements, or food products that would potentially affect the metabolism of caffeine within one week of the start of the study. The subjects were instructed not to consume more than five units of xanthine-containing products, to smoke more than five cigarettes per day or to consume more than 21 (male subjects) or 14 (female subjects) units of alcohol per week. The subjects had to refrain from consuming xanthine-containing products (within days of caffeine administration and throughout the study day) or alcohol and cigarettes (within 12 hours of caffeine administration and throughout the study day). On study days, the subject's use of medications, alcohol, and/or drugs was questioned, and a urine drug screen, pregnancy test (where applicable), and alcohol breath test were performed prior to the start of any study-related procedures. This study was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands.

Study design

ADOLESCENT STUDY

This study was a randomized, double-blind, placebo-controlled, two-way crossover study. Each subject received two cups of espresso coffee containing approximately 135 mg caffeine (according to the manufacturer) or two cups of decaffeinated espresso coffee containing <10 mg caffeine on two separate study days. To increase palatability, sucrose and dehydrated milk were available. This dose of caffeine (135 mg) was expected to block adenosine receptors and – to a small extent – phosphodiesterases[16], while minimizing undesired adverse events. Decaffeinated coffee was chosen as a placebo to make the two intervention arms as comparable as possible with respect to expectancy, sensory effects (due to the similar taste of caffeinated and decaffeinated coffee) and the presence of biologically active substances other than caffeine[17]. The beverages were administered following a minimum fasting period of four hours. A standardized light breakfast and a standardized lunch were offered 2.5 hours and 4.5 hours after coffee consumption, respectively. Water was provided ad libitum. The subjects were confined to the clinical research unit for approximately six hours after caffeine consumption.

Based on previous studies conducted at CHDR, a sample size of 16 subjects was sufficient to detect a 10% in adaptive tracking and a 5% change in saccadic peak velocity with 80% power (two-sided test, alpha = 0.05) using a randomized Williams square design.

KINETIC ADULT STUDY

This study was a randomized, open-label, two-way crossover study. Each subject received two cups of espresso coffee containing approximately 135 mg caffeine or one capsule containing 200 mg coffeine (Pharmaline, Oldenzaal, the Netherlands) on two separate study days. The dose of 200 mg in capsule form was chosen based on a PK model using data from the study in healthy adolescents and reported data in adults (Figure 1). The capsule was administered with approximately two cups of water. Where applicable, female subjects were studied while taking their oral contraceptive (i.e., not during the stop week). The caffeinated beverage or capsule was administered following a minimum fasting period of four hours. A standardized light breakfast, lunch and dinner were offered 2 hours, 4^2/3 hours and 9 hours after caffeine administration. Water (200 ml) was given every two hours after the caffeine

administration to maintain all subjects on a consistent hydration schedule. The subjects were confined to the clinical research unit for approximately 11 hours after caffeine administration.

For this study, no formal power calculation was performed. However, based on previous experience, we expected that a sample size of six subjects would be sufficient for determining the pharmacokinetic parameters and the saliva-to-plasma ratios.

In both the adolescent and adult studies, the wash-out period between study days was ≥3 days, and caffeine (or placebo) was administered at the same time of day to minimize any confounding effect of circadian rhythm.

Pharmacokinetics

In adolescents, saliva was collected in Salivette tubes (Sarstedt, Numbrecht, Germany) prior to caffeine administration (to confirm compliance) and every 15 minutes thereafter for 120 minutes, then every 60 minutes until the 240-minute time point, then every two hours until the 360-minute time point. In the adult study, the post-caffeine sampling time points were determined based on a PK model using data from the study in healthy adolescents and reported data in adults (see Figure 1); samples were collected prior to caffeine administration, 10, 30, and 60 minutes after administration, and 2, 4, 7, and 11 hours after administration. Because caffeine saliva PK can be complicated by pH partitioning, saliva was collected actively, as stimulated saliva flow leads to a stronger correlation between plasma and saliva caffeine levels[18,19]. At each time point, the subjects were instructed to insert three Salivette in their mouth and move it around the oral cavity for two minutes. Both saliva pH and saliva flow rate were measured to determine whether any fluctuation in these parameters might account for any remaining variability in the S/P caffeine ratio. Immediately after collecting and weighing the saliva (to measure salivary flow), the swabs were centrifuged at 2000x*g* for 10 minutes at 4 °C. The three saliva samples per subject were then pooled, and pH was measured using a Symphony pH meter (model SP70P, VWR Scientific) equipped with a pH/Redox electrode (range: -2.000 to 19.999; relative accuracy: ±0.002). The saliva was then transferred to 2-ml tubes (Sarstedt) and immediately stored at -80°C.

For PK analyses, venous blood samples were obtained from the adult subjects via an indwelling catheter four minutes after the start of each saliva sample collection. Blood samples were collected in 6-ml EDTA tubes and kept on ice. The samples were centrifuged (2000x*g* for 10 minutes at 4°C) as soon as possible after collection, but within 30 minutes of collection. The saliva supernatants and plasma samples were stored at -20°C until analysis.

Caffeine concentrations in the saliva samples obtained during the adolescent study were measured at Erasmus Medical Center (Rotterdam, the Netherlands). Caffeine concentrations in the saliva and plasma samples obtained during the adult study were measured at the Academic Medical Center (Amsterdam, the Netherlands). In both laboratories, a validated high-performance liquid chromatography method was used[19]. Samples were either analyzed immediately upon arrival at the laboratory or stored at 4°C until analysis (within 24 hours). The limit of quantification was 0.2 mg/L in a sample volume of 100 µl.

Pharmacodynamics

An extensive CNS battery was implemented to determine which functional CNS domains are affected by caffeine. All pharmacodynamics (PD) measurements—with exception of the visual and verbal learning task (VVLT)—were performed at the same time points as the saliva collection time points (see above). VVLT was administered approximately 45 and 150 minutes after caffeine administration in order to assess immediate and delayed recall. The PD measurements were performed in a quiet room with ambient lighting, with only one subject in the room per session. Prior to the first study day, the subjects were familiarized with the experimental procedure and performed a practice testing session to minimize potential learning effects during the study days. The tests were performed as described below.

SACCADIC AND SMOOTH PURSUIT EYE MOVEMENT

Saccadic and smooth pursuit eye movements were recorded as described previously[20–22]; in adults, these movements can be affected by many drugs that act upon the CNS, including GABAergic[23], serotonergic[24], noradrenergic[25,26], and dopaminergic drugs. For this test, we use a computer-based system for data recording and analysis (Cambridge Electronics Design, Cambridge, UK), a Nihon Kohden device for stimulus display, signal collection, and signal amplification (Nihon Kohden Corporation, Tokyo, Japan), and single-use surface electrodes (Medicotest N-00-S, Olstykke, Denmark). The average values for latency (i.e., response time), peak saccadic velocity, and the inaccuracy of all artifact-free saccades were used as parameters for quantifying saccadic eye movements. Saccadic inaccuracy was calculated as the absolute difference between the stimulus angle and the corresponding saccade, expressed as a percentage of the stimulus angle. A higher percentage reflects poorer performance on the eye movement test. For smooth pursuit, the target moved in a sinusoidal pattern over 20 degrees of eyeball rotation at a frequency ranging from 0.3–1.1 Hz. The primary parameter was the percentage of time that the eyes pursued the target smoothly.

BODY SWAY

The body sway metric records body movements in a single plane, thus providing a measure of postural stability. A variety of CNS-active drugs induce a change in body sway, including GABAergic drugs[27,28], cannabinoids such as tetrahydrocannabinol[29], and CNS stimulants such as methylphenidate[30,31]. Body sway was measured as described previously[32].

ADAPTIVE TRACKING

Adaptive tracking measures visuomotor coordination and vigilance and was performed as originally described by Borland and Nicolson[33]. This test was adapted for use on a personal computer. The adaptive tracking test has been used previously to measure the CNS effects of alcohol, sleep deprivation, and a wide range of CNS-active drugs, including stimulants such as methylphenidate[30,31]. The average performance during a 3-minute testing period was used for statistical analysis.

LEFT/RIGHT DISTRACTION TASK

A parametric version of the color-word response conflict task[34] was used to measure intervention-induced inhibition[35]. This task has been used previously to measure the effects of several compounds, including antipsychotics[36]. In this task, the word 'Left' or 'Right' was displayed on either the left or right side of a computer screen. The subject was instructed to respond quickly by pressing the button corresponding to the location of the word, irrespective of the word's meaning. The output parameters included response time and response accuracy as a function of task difficulty.

FINGER TAPPING

The finger tapping test evaluates motor activation and fluency and was adapted from the Halstead-Reitan Neuropsychological Battery[37]. During the test, the rate of tapping the index finger on the dominant hand was measured; a session comprised five 10-second tests. The subject was instructed to tap the index finger as rapidly as possible on the space key of a computer keyboard. The output measure was mean tapping rate and standard deviations were used for statistical analysis.

VISUAL AND VERBAL LEARNING TASK

The VVLT has been used previously to identify the CNS effects of various compounds, including benzodiazepines[38], antidopaminergic agents[32], and cannabinoid drugs[29]. This task was performed as described previously[32]. The primary outcomes for the immediate and delayed word-recall tasks were the numbers of correct responses; the primary outcomes for the delayed word recognition task were the number of correct items and the mean response time for correct responses. Learning was measured using the change in reproduced words with three consecutive memorization trials, decay from the change in reproduced words after a time delay (delayed word recall versus the last trial

of immediate word recall), and retrieval (the difference between delayed word recognition and delayed word recall).

ATTENTION SWITCHING TASK

This task was used to measure executive control. CHDR uses an adaptive version of a task-switching design with a Go/no Go task as described by Wylie and colleagues (2003). The output parameters are response time and error-rate.

PUPIL SIZE MEASUREMENT

Measurement of the pupil size was done by taking a picture from both eyes simultaneously while the subject was seated on a chair with his head fixed in the head support system. From the pictures the ratio between the pupil - and iris diameter was calculated

BLOOD PRESSURE AND HEART RATE

Blood pressure was measured using a Nihon Kohden BSM-1101K automated oscillometric monitor, a Pressmate BP 8800 (Colin), or a Dash 4000 monitor. Heart rate was measured using oscillometry.

Statistical analysis and pharmacometrics

PHARMACODYNAMICS

The PD endpoints of the adolescent study were analyzed using mixed-model analyses of variance (using the SAS PROC MIXED program). Subject, subject by treatment, and subject by time were random effects; treatment, test day, time, and treatment by time were fixed effects; and the average baseline value was a covariate. Contrasts were estimated within the overall treatment effect, and contrasts between treatments >360 min were calculated. Body sway and attention switch task variables were log-transformed, analyzed, and then back-transformed (the results are presented as percent change). The attention switch error rate was transformed by adding 1 to all data points in order to avoid log-transforming a value of 0. Because the VVLT parameters were assessed only once for each treatment arm, the raw test scores were evaluated. Data analyses included an analysis of variance with baseline correction. This study tested the following null hypothesis: 'there is no difference between caffeine and placebo'.

Pharmacometrics

DATA

Exploratory individual and summary concentration-time profiles were generated in order to identify potential outliers, to understand the influence of censoring concentrations below the limit of quantification (LOQ), and to yield information regarding the base structural model. Concentration-time curves were plotted for the plasma and saliva samples in order to identify indirect relationships (e.g., time shifts). Samples below LOQ before T_{max} were set to zero.

MODELING STRATEGY

Pharmacometrics were performed using nonlinear mixed-effect modeling (NONMEM version 7.2.0[39], Icon Development Solutions, Ellicott City, MD, USA, 2009). First-order conditional estimation with interaction (FOCEI) was used for the estimation, with a convergence criterion of five significant digits in the parameter estimations. NONMEM reports the objective function value (OFV), which is the -2 times log likelihood (-2LL). Models were compared using the likelihood ratio test with the assumption that the difference in -2LL is Chi-square distributed, with degrees of freedom determined by the number of additional parameters in the more complex model. Therefore, with a decrease in OFV of at least 6.63 points, a model with one additional parameter is considered superior to its parent model ($p<0.01$). Different models with increasing complexity were compared to identify the simplest model that described the data adequately. Graphical analysis was used to assess model performance while developing the model.

The following goodness-of-fit plots were prepared: observed concentration (the dependent variable, or DV) versus population concentration (PRED); DV versus individual predicted concentration (IPRED); weighted residuals with interaction (CWRESI) versus IPRED; CWRESI versus time after dose; combined PRED, IPRED, and DV versus time (per individual) and distribution of inter-individual variability (IIV) estimates (ETA). Covariates were analyzed using a stepwise approach. For the visual predictive check (VPC), dosing regimens were simulated as performed in the adolescent and adult studies based on the distribution of lean body mass (LBM[40]) in the data sets. The best PK and PK/PD models were selected based on the likelihood ratio test, diagnostic plots, VPC, parameters, and precision in parameter estimates. The relative standard error (RSE) was calculated and used to derive the uncertainty in the parameter estimates; RSE <10% was considered acceptable. Preparing the NONMEM input file and processing of the model results (i.e., preparing tables and graphs) was performed using R version 2.12.0[41]. VPC was performed using the lsoda function from the deSolve library (version 1.8.1) and the function mvrnorm from the MASS library (version 7.3-8) by simulating 1000 replications of the best models and a simulation data set (up to 12 hours). The median prediction and 95% prediction interval were calculated for each simulated time point and compared to the observations.

POPULATION PK MODEL DEVELOPMENT

First, a population plasma PK model for caffeine was developed using the plasma data obtained from the adult study. One-compartment and two-compartment structural models, as well as different compartmental and elimination sub-models, were tested. All models used a first-order process to describe the oral absorption of caffeine. Interindividual variability (IIV) was assessed separately for each PK parameter using a stepwise bottom-up approach. Correlations between the IIV of each parameter were determined graphically. When a correlation was found to be significant (either by shape or Pearson's correlation coefficient), covariance between the terms was assessed by applying an omega block on selected parameters and accepted based on the results of a likelihood ratio test. For the parameter estimation, shrinkage <30% was considered acceptable[42]. Proportional and combined error structures were evaluated to best describe the residual error. Based on graphical identification, the most promising covariates were tested in the model; these covariates were then included based on a decrease in OFV in a stepwise manner (forward inclusion of covariates, followed by a backward elimination step). A plasma-saliva PK model for caffeine was then developed using the saliva data collected from the study in adults. All plasma PK parameters were fixed to the individual estimates that were derived from the best plasma PK model. The simulated individual plasma concentration-time profiles consequently drive the saliva caffeine concentrations, for which different model structures were explored (e.g., a linear effect model or an effect compartment). The model was developed as described for the best PK plasma model (e.g., by incorporating IIV, residual error, and covariates). The best model was validated by performing a VPC of the simulated saliva concentrations in adolescents.

Results

Subjects

A total of 16 adolescent subjects (10 males, 6 females) 15-18 years of age (mean: 16.8 years) were enrolled in 2009, and a total of 7 adult subjects (5 males, 2 females) 19-24 years of age (mean: 21.4 years) were enrolled in 2012. All adolescent subjects were attending pre-university secondary education (in Dutch, *Voortgezet Wetenschappelijk Onderwijs* or VWO). One of the male adult volunteers discontinued the study after the first study day (a caffeine capsule) for reasons unrelated to the study and was replaced. The mean weight and height of the adolescent subjects were 69 kg (range: 50-85 kg) and 1.80 m (range: 1.65-1.93 m), respectively; the mean weight and height of the adult subjects were 77 kg (range: 58-90 kg) and 1.82 m (range: 1.70-1.98 m), respectively. None of the adolescents reported using cigarettes; one adult subject reported smoking four cigarettes per day. One female adult subject used an oral contraceptive

during the study. In addition, one adolescent subject used paracetamol during the study (a single 500-mg dose taken 2.5 hours prior to dosing on the first study day.) The mean daily consumption of xanthine-containing products in the preceding months was 2.6 (range: 0-5) standard units for the adolescents and 1.7 (range: 0-4) standard units for the adults.

Pharmacodynamic results

NEUROPHYSIOLOGICAL PARAMETERS

Caffeine administration significantly increased adaptive tracking performance and saccadic peak velocity and accelerated saccadic reaction time (Table 1, Figure 2 and Figure 3). No significant effect was observed with respect to body sway, saccadic eye movement (response latency), and tapping. In addition, no significant effect was observed with respect to parameters related to memory (Table 2).

Attention switch task error rate and response time (pre-switch trial, #3) were both significantly affected by caffeine (Table 3, Figure 4). In the distraction task, the response times for incorrect responses were shorter than the response times for correct responses (Table 3).

Neither systolic blood pressure nor pupil size differed significantly between the caffeine and placebo groups (Table 4).

Pharmacometrics analysis

POPULATION PK PLASMA MODEL DEVELOPMENT

A total of 90 PK observations from plasma samples were collected from six adult subjects. One data point obtained prior to T_{max} was below LOQ and was set to zero.

A two-compartment model with a dose depot and first-order absorption and elimination kinetics described the caffeine plasma concentrations in the adult subjects best. The parameter estimates, relative standard error (RSE) and IIV are summarized in Tables 5 and 6. The parameter for lag time after oral administration (ALAG1) was fixed at a value of 0.23 hours (the time point before the first PK sample), as this parameter was needed in order to describe the absorption phase; however, the number of data points in the absorption phase was not sufficient to accurately quantify the two necessary absorption parameters (ka and ALAG). For the beverage treatment group, V2 (the distribution volume for the central compartment) was corrected by the fractional bioavailability, as bioavailability differs between beverage and oral treatment. To facilitate fitting of the model, the initial parameter estimates and boundaries for the parameters estimates were selected based on published values. The residual error model included combined proportional and additive errors. IIV was identified on ka (the absorption rate constant) – with different variability between the two treatments – on clearance, on ke and on the correction fraction of V2 for the beverage group. Two parameter estimates with large confidence intervals (ka and Q) were modeled as an exponent in order to prevent negative – and thus physiologically impossible – values for these parameters. LBM was implemented in the model as a covariate of V2 in normalized power function (Eq. 1).

$$P_i = P_p \times (COV/COV_{median})^k \qquad \text{Eq. 1}$$

where P_i; individual parameter estimate, P_p; typical (population) value, COV; individual covariate value, COV_{median}; normalization value for covariate, k; parameter estimate for the exponent.

In the best PK model, the population concentrations and individual predicted concentrations were close to the observed values. In addition, we observed no apparent concentration-dependent or time-dependent bias (Figures 5 and 6).

POPULATION PK SALIVA MODEL DEVELOPMENT

A total of 614 PK observations from saliva samples were obtained from 6 adult and 16 adolescent subjects. All data points collected prior to T_{max} and below LOQ were set to zero.

The adult data were described best by a PK model that described saliva concentration as a fraction of the plasma concentration. However, this model was

valid only for observations made >1 hour after the dose. Prior to the 1-hour time point, the relationship between the plasma and saliva concentrations was too complex to develop a physiologically plausible model, regardless of whether the caffeine was delivered as a beverage or capsule (Figure 7). Therefore, only saliva concentrations measured >1 hour after dosing were included in the analysis. The estimated parameter was the S/P ratio (α; 0.68). Because incorporating IIV into the estimated ratio did not improve the model, the relationship between saliva pH or flow and the S/P ratio could not be evaluated adequately. The residual error model included an additive error. The best model accurately described saliva concentrations for both capsule and beverage dosages in adults for >1 hour after dosing (Figures 8 and 9). The best model also accurately described saliva concentrations in adolescents from >1 hour after dosing, although the saliva concentrations in adolescents were slightly under-predicted (Figure 10).

Discussion

Although the behavioral and psychomotor effects of caffeine have been studied extensively[43-45], they have not been investigated in adolescents. Because caffeine can affect adolescents and adults differently[2], results obtained in adults cannot necessarily be translated to adolescents. This is the first study to examine the CNS effect profile of a single oral dose of caffeine (a caffeinated beverage) specifically in adolescents. In adolescents, caffeine had significant effects on task parameters related to attention, visuomotor coordination (adaptive tracking task), and alertness (saccadic peak velocity). Caffeine also induced cognitive effects, including an increase in error rate in the attention switch task.

Because previous studies have demonstrated that saliva is an appropriate matrix for estimating caffeine concentration in plasma[46,47], we measured caffeine concentration in saliva samples. To correlate caffeine's effects in adolescents with the estimated plasma caffeine concentration, we performed a PK study in adults and determined the S/P ratio for caffeine concentration. Plasma caffeine concentration in adults was described best as a two-compartment model with a dose depot, first-order absorption kinetics, and first-order elimination kinetics. Although some published reports suggest that caffeine PK in plasma can be described using a one-compartment model[48], some individual concentration-time profiles suggested two-compartment properties. Therefore, a two-compartment model was tested and was found to be superior to the one-compartment model. The apparent clearance and distribution volume were consistent with published values in adults[48-50] and were estimated here with high precision. For the caffeinated beverage, the dose identified by the model was lower (by a factor of 0.67) than the dose indicated by the manufacturer (135 mg) and varied among the subjects. With respect to ka, the relative standard error of the population values was high, which could be attributed—at least in part—to the inclusion of PK data obtained after the administration of different formulation types, the low number of observations collected during the absorption phase, and caffeine's moderately fast absorption rate. Two IIV terms were required for the absorption rate (one for each formulation), and an arbitrarily fixed lag time of 0.23 hours was needed in order to stabilize the model. IIV for the absorption rate was large for both formulations compared to IIV terms for other PK parameters, and this likely contributed to unexplained variability in the plasma C_{max} and T_{max} values. In addition, this high degree of variability necessitated the use of a relatively large number of parameters in order to describe the data accurately. Thus, although this population PK model adequately describes the variability in data obtained in this study population, it should be used with caution when used for predictive purposes. Consistent with previous studies in adults[50], we also observed IIV in the apparent clearance and apparent distribution volume (in the beverage group only). Additionally, IIV was identified in inter-compartmental clearance in the beverage group only (as this parameter is related to the apparent distribution volume) and the elimination rate constant. Lean body mass-dependent variability was identified for the volume of the central compartment. Other potential covariates, including smoking status[50] and

oral contraception[51], could not be evaluated, as only one subject smoked and only one female subject was taking birth control pills at the time of the study.

The population PK model for plasma caffeine was extended by including a population model that described caffeine saliva concentration in adults >1 hour after administration; the model showed that saliva concentration was a fraction (0.68) of plasma concentration, which is consistent with previous studies in neonates[19] and adults[20]. The relationship between plasma and saliva caffeine concentrations could not be described as linear at earlier time points. Several studies in healthy adults reported a time-dependent S/P ratio for caffeine concentration, with a higher initial ratio that was followed by a decrease in ratio at later time points[52-54]. This time-dependent phenomenon has been attributed to fluctuations in the arteriovenous blood concentration[54] and pH partitioning[53]. In our PK study in adults, although saliva pH and flow were measured at each sampling time point, they could not be quantified as covariates in our model.

A slight under-prediction was observed with respect to simulations of saliva concentration in adolescents. This may have been due to several factors in addition to the abovementioned limitations in the plasma PK model. Possible study-related factors include a variable—and therefore potentially slightly higher—dose in the adolescent study. Contamination also likely played a role in the early time points; however, this was likely not a factor at time points >1 hour after administration. In addition, the simulations were based on the distribution of LBM in the adolescent data set, which included more male subjects (with generally higher LBM) than female subjects, perhaps explaining why the observed saliva concentrations were in the upper half of the 95% prediction interval for the majority of female subjects. The formula that we used to calculate LBM[18] may have caused an under-estimation of LBM in some of the male adolescents. This formula uses weight and body mass index (height/weight^2) and is based on data from adults, as no validated equation has been developed for adolescents ≥15 years of age. However, in a recent study, an equation for LBM that included only height and weight resulted in the systematic under-estimation of LBM in male adolescents between 13 and 15 years of age[55]. Finally, other factors could be related to differences between adolescents and adults, although an age-dependent difference in the S/P ratio is unlikely, as reports suggest similar ratios across various ages[19,20]. Relative under-prediction of adolescent saliva concentrations may reflect differences in PK variability between adolescents and adults. A pubertal stage dependent decrease in CYP1A2-mediated caffeine clearance has been described in healthy adolescents[56], and weight-normalized clearance values of caffeine's active metabolite theophylline in sexually mature adolescents have been reported to be lower (~45%) than those in prepubescent individuals[57]. Finally, because adolescents of the same age range can have different levels of physiological maturation, caffeine clearance is likely to vary widely among adolescents, resulting in a broader 95% prediction interval (and thus also higher concentrations at the highest percentile) than previously estimated using data obtained from adults.

Because the S/P ratio for caffeine could only be described accurately >1 hour after administration, the resulting PK model was not sufficient for developing a PK/PD model using adolescent data alone. Significant effects in adolescents were seen one hour of administration as well as 3-4 hours after administration. For example, caffeine's effects on the adaptive tracking task peaked soon after administration, which may be explained by caffeine's low EC_{50} (half-maximal effective concentration). Alternatively, caffeine could facilitate task learning (learning effects) rather than task performance. Caffeine's effects that occurred within one hour were not suitable for developing a PK/PD model based on simulated plasma caffeine concentration; however, these effects could be included in a PK/PD model that is based on measured saliva caffeine concentration. With compounds that diffuse freely, including caffeine, the salivary concentration provides a better estimate of the drug's cellular concentration in organs than in peripheral venous blood[54]; thus, saliva concentrations may be superior to plasma concentrations for developing a model. However, such a model must be corrected for possible contamination (particularly in individual samples collected directly after administration of a caffeinated beverage). Unfortunately, contamination could not be estimated

in one of the sub-models due to the limited number of observations. Caffeine's effects that occur >1 hour after administration could be used to develop a PK/PD model. For example, caffeine's effects on saccadic peak velocity occurred 3-4 hours after administration, and several of caffeine's effects occur with a time delay relative to changes in plasma concentrations, with an equilibration half-life of 20-50 minutes[14,15]. However, whether these late effects can be attributed to caffeine's distribution time into tissues, post-receptor changes, or the formation of active metabolites (such as theophylline, which acts as an adenosine receptor antagonist[58]) remains unclear.

In our study of adolescents, a low to moderate dose of caffeine of approximately 135 mg was chosen because this dose can significantly block adenosine receptors[16]. However, the actual dose of caffeine that was absorbed from the beverage was likely lower than 135 mg, as the plasma model identified a dose of 90 mg in the adults who ingested the same caffeinated beverage. Despite this decreased effective dose, caffeine-related effects were observed in parameters regarding alertness and reaction time in adolescents, effects that are more commonly observer following a low to moderate dose of caffeine in adults[59,60]. It is therefore likely that larger changes would occur at higher caffeine doses for some outcome parameters such as body sway, response times (of saccadic eye movements and the distraction task), and blood pressure; parameters that changed slightly but did not reach statistical significance. For example, a dose-dependent increase in diastolic blood pressure was reported in a recent study in adolescents[61]. In contrast to previous findings in adults, caffeine's effects on parameters in the attention switch task included an increase in error rate, but no effect on response time in the switch trial. Performance in repeated-task trials is typically better than performance in 'switch' trials. Caffeine has been shown to reduce switch error rates and/or response time relative to placebo[62,63], and coffee consumption has been suggested to improve task-switching performance by enhancing anticipatory processing (e.g., task set updating), presumably via caffeine's neurochemical effects on the dopaminergic system[62]. In addition, in our study the response time for incorrect responses was shorter than the response time for correct responses in the distraction task, which may suggest that impulsivity increases after caffeine intake in adolescents. Neuroimaging studies have shown that task switching involves an extensive neural network, including lateral prefrontal and parietal cortical regions, the pre-supplementary motor area, and the anterior cingulate cortex[64-69], neural regions that functionally mature during adolescence[70]. In addition, animal studies suggest that the dopaminergic system undergoes major changes during adolescence (for review, see[71]); in addition, other CNS stimulants (for example, amphetamine) can have age-dependent effects on impulsivity[72]. However, as the switch trial is not designed to evaluate impulsivity, it remains to be determined if caffeine indeed increases impulsivity in adolescents.

In conclusion, we report that a low dose of caffeine induces significant effects on parameters regarding alertness and reaction speed in adolescents, despite an expected ceiling effect on several parameters among this healthy, alert population. Whether these low-dose effects of caffeine are stronger in adolescents than in adults remains to be determined. Caffeine did not improve task-switching performance, but it may increase impulsivity. Finally, because the S/P ratio for caffeine in adults could only be described accurately >1 hour after receiving the dose, and because the level of contamination after consuming the caffeinated beverage could not be quantified, the early effects of caffeine in adolescents were not suitable for developing a PK/PD model.

REFERENCES

1 Nehlig A. Are we dependent upon coffee and caffeine? A review on human and animal data. *Neurosci Biobehav Rev.* 1999;23:563-576.
2 Temple JL. Caffeine use in children: what we know, what we have left to learn, and why we should worry. *Neurosci Biobehav Rev.* 2009;33:793-806.
3 Calamaro CJ, Mason TB, Ratcliffe SJ. Adolescents living the 24/7 lifestyle: effects of caffeine and technology on sleep duration and daytime functioning. *Pediatrics.* 2009;123:e1005-e1010.
4 Harnack L, Stang J, Story M. Soft drink consumption among us children and adolescents: nutritional consequences. *J Am Diet Assoc.* 1999;99:436-441.
5 Castellanos FX, Rapoport JL. Effects of caffeine on development and behavior in infancy and childhood: a review of the published literature. *Food Chem Toxicol.* 2002;40:1235-1242.
6 Rapoport JL, Jensvold M, Elkins R, Buchsbaum MS, Weingartner H, Ludlow C, et al. Behavioral and cognitive effects of caffeine in boys and adult males. *J Nerv Ment Dis.* 1981;169:726-732.
7 Marin MT, Zancheta R, Paro AH, Possi AP, Cruz FC, Planeta CS. Comparison of caffeine-induced locomotor activity between adolescent and adult rats. *Eur J Pharmacol.* 2011;660:363-367.
8 Rhoads DE, Huggler AL, Rhoads LJ. Acute and adaptive motor responses to caffeine in adolescent and adult rats. *Pharmacol Biochem Behav.* 2011;99:81-86.
9 Bolanos CA, Glatt SJ, Jackson D. Subsensitivity to dopaminergic drugs in periadolescent rats: a behavioral and neurochemical analysis. *Brain Res Dev Brain Res.* 1998;111:25-33.
10 Laviola G, Wood RD, Kuhn C, Francis R, Spear LP. Cocaine sensitization in periadolescent and adult rats. *J Pharmacol Exp Ther.* 1995;275:345-357.
11 Chen YI, Choi JK, Xu H, Ren J, Andersen SL, Jenkins BG. Pharmacologic neuroimaging of the ontogeny of dopamine receptor function. *Dev Neurosci.* 2010;32:125-138.
12 Levant B, Zarcone TJ, Davis PF, Ozias MK, Fowler SC. Differences in methylphenidate dose response between periadolescent and adult rats in the familiar arena-novel alcove task. *J Pharmacol Exp Ther.* 2011;337:83-91.
13 Chen YI, Choi JK, Xu H, Ren J, Andersen SL, Jenkins BG. Pharmacologic neuroimaging of the ontogeny of dopamine receptor function. *Dev Neurosci.* 2010;32:125-138.
14 Shi J, Benowitz NL, Denaro CP, Sheiner LB. Pharmacokinetic-pharmacodynamic modeling of caffeine: tolerance to pressor effects. *Clin Pharmacol Ther.* 1993;53:6-14.
15 Seng KY, Teo WL, Fun CY, Law YL, Lim CL. Interrelations between plasma caffeine concentrations and neurobehavioural effects in healthy volunteers: model analysis using NONMEM. *Biopharm Drug Dispos.* 2010;31:316-330.
16 Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev.* 1999;51:83-133.
17 Hindmarch I, Quinlan PT, Moore KL, Parkin C. The effects of black tea and other beverages on aspects of cognition and psychomotor performance. *Psychopharmacology (Berl).* 1998;139:230-238.
18 Choo RE, Huestis MA. Oral fluid as a diagnostic tool. *Clin Chem Lab Med.* 2004;42:1273-1287.
19 de Wildt SN, Kerkvliet KT, Wezenberg MG, Ottink S, Hop WC, Vulto AG, et al. Use of saliva in therapeutic drug monitoring of caffeine in preterm infants. *Ther Drug Monit.* 2001;23:250-254.
20 van Steveninck AL, Schoemaker HC, Pieters MS, Kroon R, Breimer DD, Cohen AF. A comparison of the sensitivities of adaptive tracking, eye movement analysis and visual analog lines to the effects of incremental doses of temazepam in healthy volunteers. *Clin Pharmacol Ther.* 1991;50:172-180.
21 Van Steveninck AL. Methods of assessment of central nervous system effects of drugs in man. State University Leiden; 1994.
22 Van Steveninck AL, van Berckel BN, Schoemaker RC, Breimer DD, van Gerven JM, Cohen AF. The sensitivity of pharmacodynamic tests for the central nervous system effects of drugs on the effects of sleep deprivation. *J Psychopharmacol.* 1999;13:10-17.
23 de Visser SJ, van der Post JP, de Waal PP, Cornet F, Cohen AF, van Gerven JM. Biomarkers for the effects of benzodiazepines in healthy volunteers. *Br J Clin Pharmacol.* 2003;55:39-50.
24 Gijsman HJ, van Gerven JM, Verkes RJ, Schoemaker RC, Pieters MS, Pennings EJ, et al. Saccadic peak velocity and EEG as end-points for a serotonergic challenge test. *Hum Psychopharmacol.* 2002;17:83-89.
25 van der Post JP, de Visser SJ, Schoemaker RC, Cohen AF, van Gerven JM. Pharmacokinetic/pharmacodynamic assessment of tolerance to central nervous system effects of a 3 mg sustained release tablet of rilmenidine in hypertensive patients. *J Psychopharmacol.* 2004;18:221-227.
26 Kemme MJ, vd Post JP, Schoemaker RC, Straub M, Cohen AF, van Gerven JM. Central nervous system effects of moxonidine experimental sustained release formulation in patients with mild to moderate essential hypertension. *Br J Clin Pharmacol.* 2003;55:518-525.
27 de Haas SL, de Visser SJ, van der Post JP, Schoemaker RC, van DK, Murphy MG, et al. Pharmacodynamic and pharmacokinetic effects of MK-0343, a GABA(A) alpha2,3 subtype selective agonist, compared to lorazepam and placebo in healthy male volunteers. *J Psychopharmacol.* 2008;22:24-32.
28 de Visser SJ, van der Post JP, de Waal PP, Cornet F, Cohen AF, van Gerven JM. Biomarkers for the effects of benzodiazepines in healthy volunteers. *Br J Clin Pharmacol.* 2003;55:39-50.
29 Zuurman L, Roy C, Schoemaker RC, Hazekamp A, den HJ, Bender JC, et al. Effect of intrapulmonary tetrahydrocannabinol administration in humans. *J Psychopharmacol.* 2008;22:707-716.
30 Hay JL, van Gerven JMA. A meta-analysis of pharmacodynamic testing with the NeuroCart used in the early phase drug development of antidepressants, stimulants, and CNS depressant agents. Dutch Medicines Days, Lunteren, The Netherlands. 2012.
31 van Gerven JMA, Hay JL. A meta-analysis of pharmacodynamic testing with the NeuroCart used in early phase drug development. NCDEU meeting, Boca Raton, Florida, USA. 2011.
32 Liem-Moolenaar M, Gray FA, de Visser SJ, Franson KL, Schoemaker RC, Schmitt JA, et al. Psychomotor and cognitive effects of a single oral dose of talnetant (SB223412) in healthy volunteers compared with placebo or haloperidol. *J Psychopharmacol.* 2010;24:73-82.
33 Borland RG, Nicholson AN. Visual motor co-ordination and dynamic visual acuity. *Br J Clin Pharmacol.* 1984;18 Suppl 1:69S-72S.
34 Stroop JR. Studies of interference in serial verbal reactions. Journal of Experimental Psychology 18[6], 643-662. 1935.
35 Laeng B, Lag T, Brennen T. Reduced Stroop interference for opponent colors may be due to input factors: evidence from individual differences and a neural network simulation. *J Exp Psychol Hum Percept Perform.* 2005;31:438-452.
36 Cuesta MJ, Peralta V, Zarzuela A. Effects of olanzapine and other antipsychotics on cognitive function in chronic schizophrenia: a longitudinal study. *Schizophr Res.* 2001;48:17-28.

37 Yeudall LT, Reddon JR, Gill DM, Stefanyk WO. Normative data for the Halstead-Reitan neuropsychological tests stratified by age and sex. *J Clin Psychol.* 1987;43:346-367.
38 de Haas SL, de Visser SJ, van der Post JP, De SM, Schoemaker RC, Rijnbeek B, et al. Pharmacodynamic and pharmacokinetic effects of TPA023, a GABA(A) alpha(2,3) subtype-selective agonist, compared to lorazepam and placebo in healthy volunteers. *J Psychopharmacol.* 2007;21:374-383.
39 Beal. NONMEM User's Guides. (1989-2009). 2009.
40 Janmahasatian S, Duffull SB, Ash S, Ward LC, Byrne NM, Green B. Quantification of lean bodyweight. *Clin Pharmacokinet.* 2005;44:1051-1065.
41 R Development Core Team. R: A Language and Environment for Statistical Computing. 2010. Vienna, Austria, R Foundation for Statistical Computing.
42 Savic RM, Karlsson MO. Importance of shrinkage in empirical bayes estimates for diagnostics: problems and solutions. AAPS *J.* 2009;11:558-569.
43 Lieberman HR, Wurtman RJ, Emde GG, Roberts C, Coviella IL. The effects of low doses of caffeine on human performance and mood. *Psychopharmacology (Berl).* 1987;92:308-312.
44 Smith A. Effects of caffeine on human behavior. *Food Chem Toxicol.* 2002;40:1243-1255.
45 Smith A, Sutherland D, Christopher G. Effects of repeated doses of caffeine on mood and performance of alert and fatigued volunteers. *J Psychopharmacol.* 2005;19:620-626.
46 Perera V, Gross AS, Xu H, McLachlan AJ. Pharmacokinetics of caffeine in plasma and saliva, and the influence of caffeine abstinence on CYP1A2 metrics. *J Pharm Pharmacol.* 2011;63:1161-1168.
47 Alkaysi HN, Salem MS, el-Sayed YM. High performance liquid chromatographic analysis of caffeine concentrations in plasma and saliva. *J Clin Pharm Ther.* 1988;13:109-115.
48 Seng KY, Teo WL, Fun CY, Law YL, Lim CL. Interrelations between plasma caffeine concentrations and neurobehavioural effects in healthy volunteers: model analysis using NONMEM. *Biopharm Drug Dispos.* 2010;31:316-330.
49 Csajka C, Haller CA, Benowitz NL, Verotta D. Mechanistic pharmacokinetic modelling of ephedrine, norephedrine and caffeine in healthy subjects. *Br J Clin Pharmacol.* 2005;59:335-345.
50 Seng KY, Fun CY, Law YL, Lim WM, Fan W, Lim CL. Population pharmacokinetics of caffeine in healthy male adults using mixed-effects models. *J Clin Pharm Ther.* 2009;34:103-114.
51 Balogh A, Klinger G, Henschel L, Borner A, Vollanth R, Kuhnz W. Influence of ethinylestradiol-containing combination oral contraceptives with gestodene or levonorgestrel on caffeine elimination. *Eur J Clin Pharmacol.* 1995;48:161-166.
52 Newton R, Broughton LJ, Lind MJ, Morrison PJ, Rogers HJ, Bradbrook ID. Plasma and salivary pharmacokinetics of caffeine in man. *Eur J Clin Pharmacol.* 1981;21:45-52.
53 Zylber-Katz E, Granit L, Levy M. Relationship between caffeine concentrations in plasma and saliva. *Clin Pharmacol Ther.* 1984;36:133-137.
54 Haeckel R. Relationship between intraindividual variation of the saliva/plasma- and of the arteriovenous concentration ratio as demonstrated by the administration of caffeine. *J Clin Chem Clin Biochem.* 1990;28:279-284.
55 Foster BJ, Platt RW, Zemel BS. Development and validation of a predictive equation for lean body mass in children and adolescents. *Ann Hum Biol.* 2012;39:171-182.
56 Lambert GH, Schoeller DA, Kotake AN, Flores C, Hay D. The effect of age, gender, and sexual maturation on the caffeine breath test. *Dev Pharmacol Ther.* 1986;9:375-388.
57 Miles M, Feinstein R, Cutter G, Lawrence C. Developmental pharmacokinetics of theophylline during puberty. Clin Pharmacol Ther , 211. 1986.
58 Schwabe U, Ukena D, Lohse MJ. Xanthine derivatives as antagonists at A1 and A2 adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol.* 1985;330:212-221.
59 Kawamura N, Maeda H, Nakamura J, Morita K, Nakazawa Y. Effects of caffeine on event-related potentials: comparison of oddball with single-tone paradigms. *Psychiatry Clin Neurosci.* 1996;50:217-221.
60 Clubley M, Bye CE, Henson TA, Peck AW, Riddington CJ. Effects of caffeine and cyclizine alone and in combination on human performance, subjective effects and EEG activity. *Br J Clin Pharmacol.* 1979;7:U57-U63.
61 Temple JL, Dewey AM, Briatico LN. Effects of acute caffeine administration on adolescents. *Exp Clin Psychopharmacol.* 2010;18:510-520.
62 Tieges Z, Snel J, Kok A, Wijnen JG, Lorist MM, Richard RK. Caffeine improves anticipatory processes in task switching. *Biol Psychol.* 2006;73:101-113.
63 Einother SJ, Martens VE, Rycroft JA, De Bruin EA. L-theanine and caffeine improve task switching but not intersensory attention or subjective alertness. *Appetite.* 2010;54:406-409.
64 Braver TS, Reynolds JR, Donaldson DI. Neural mechanisms of transient and sustained cognitive control during task switching. *Neuron.* 2003;39:713-726.
65 Dove A, Pollmann S, Schubert T, Wiggins CJ, von Cramon DY. Prefrontal cortex activation in task switching: an event-related fMRI study. *Brain Res Cogn Brain Res.* 2000;9:103-109.
66 Dreher JC, Berman KF. Fractionating the neural substrate of cognitive control processes. *Proc Natl Acad Sci U S A.* 2002;99:14595-14600.
67 Kimberg DY, Aguirre GK, D'Esposito M. Modulation of task-related neural activity in task-switching: an fMRI study. *Brain Res Cogn Brain Res.* 2000;10:189-196.
68 Konishi S, Nakajima K, Uchida I, Kameyama M, Nakahara K, Sekihara K, et al. Transient activation of inferior prefrontal cortex during cognitive set shifting. *Nat Neurosci.* 1998;1:80-84.
69 Luks TL, Simpson GV, Feiwell RJ, Miller WL. Evidence for anterior cingulate cortex involvement in monitoring preparatory attentional set. *Neuroimage.* 2002;17:792-802.
70 Tamnes CK, Walhovd KB, Torstveit M, Sells VT, Fjell AM. Performance monitoring in children and adolescents: a review of developmental changes in the error-related negativity and brain maturation. *Dev Cogn Neurosci.* 2013;6:1-13.
71 Wahlstrom D, White T, Luciana M. Neurobehavioral evidence for changes in dopamine system activity during adolescence. *Neurosci Biobehav Rev.* 2010;34:631-648.
72 Hammerslag LR, Waldman AJ, Gulley JM. Effects of amphetamine exposure in adolescence or young adulthood on inhibitory control in adult male and female rats. *Behav Brain Res.* 2014;263:22-33.

TABLE 1 **Least-square means, estimates of difference and confidence intervals of neurophysiological effects of caffeine**

Parameter	Least-square means		Estimate of difference (95% CI)	p-value
	placebo	caffeine	caffeine vs. placebo	
Body Sway (mm)	272	248	-8.85% (-18.5/1.96%)	0.098
Saccadic Inaccuracy (%)	6.3	6.4	0.1% (-0.3/0.6%)	0.559
Saccadic peak velocity (deg/sec)	517	526	8.5% (2.0/15.0%)	**0.014**
Saccadic Reaction Time (sec)	0.197	0.193	-0.005% (-0.009/0%)	0.068
Smooth pursuit (%)	49.4	49.2	-0.3% (-3.1/2.6%)	0.842

95% CI = 95% confidence interval; statistically significant differences (i.e., p<0.05) are indicated in bold

TABLE 2 **Least-square means, estimates of difference and confidence intervals of effects of caffeine on cognition**

Parameter	Least-square means		Estimate of difference (95% CI)	p-value
	placebo	caffeine	caffeine vs. placebo	
Immediate word recall number correct (#3)	19.4	19.9	0.5 (-1.5/2.5)	0.6023
Delayed word recall number correct	17.5	18.1	0.6 (-1.9/3.0)	0.6330
Delayed word recognition number correct	27.1	26.4	-0.7 (-2.1/0.7)	0.3025
Response time of correct responses (word recognition)	880.2	847.0	-33.3 (-82.2/15.7)	0.1676

95% CI = 95% confidence interval; # = trial number

TABLE 3 **Mean reaction times and error rates of attention switch task, adaptive tracking, tapping and distraction task**

Parameter	Least-square means		Estimate of difference (95% CI)	p-value
	placebo	caffeine	caffeine vs. placebo	
Attention switch error rate (#1)	0.7	1.1	19.11% (1.61/39.62%)	**0.0338**
Attention switch reaction time (#3)	19525	17929	-8.17% (-11.0/-5.25%)	**<0.0001**
Adaptive tracking	26.55	28.06	1.50 (0.60/2.41)	**0.0031**
Tapping (taps/10s)	63.6	65.1	1.5 (-0.3/3.4)	0.0975
Left/right distraction number correct	31	31	-0 (-1/0%)	0.1137
Left/right distraction number incorrect	1	1	0 (-0/1%)	0.1137
Left/right distraction reaction time correct (ms)	17649	17036	-613 (-1261/34)	0.0611
Left/right distraction				
Reaction time incorrect (ms)	558	704	146 (-56/347)	0.1395

95% CI = 95% confidence interval; # = trial number

TABLE 4 **Least-square means, estimates of difference and confidence intervals of effects of caffeine on autonomic nervous system**

Parameter	Least-square means		Estimate of difference (95% CI)	p-value
	placebo	caffeine	caffeine vs. placebo	
Left pupil/Iris ratio	0.5640	0.5582	-0.006 (-0.020-0.0084)	0.3528
Right pupil/Iris ratio	0.5561	0.5598	0.0037 (-0.007/0.0147)	0.4682
Heart rate (beats per minute)	65.3	66.4	1.0 (-4.6/6.7)	0.6842
Diastolic blood pressure (mmHg)	63.7	65.1	1.4 (-0.8/3.6)	0.1857
Systolic blood pressure (mmHg)	117.2	120.8	3.6 (-0.2/7.5)	0.0611

95% ci = 95% confidence interval

TABLE 5 **Population parameter estimates of the best plasma PK model (caffeinated beverage)**

Parameter (units)	Median	RSE (%)	IIV (%)
ka (hr^{-1})	180	73.3	319
ke (hr^{-1})	0.153	12.4	27
V2/F (L)	50.7	12.7	25
Q (L/hr)	240	28.1	ND
V3 (L)	12.3	11.3	ND
ALAG1 (hr)	0.23	ND	ND
CL (L/hr)	7.73	10.4	37
k_23 (hr^{-1})	4.72	35.7	25
k32 (hr^{-1})	19.6	22.8	ND
LBM COV	0.665	11.6	ND
α	0.678	6.38	ND

Parameter uncertainties and inter-individual variability (IIV) are provided when applicable.
ND, not determined; RSE, relative standard error.

TABLE 6 **Population parameter estimates of the best plasma PK model (caffeine capsule)**

Parameter	Median	RSE (%)	IIV (%)
ka (hr^{-1})	180	73.3	2549
ke (hr^{-1})	0.153	12.4	27
V2/F (L)	30.8	4.0	ND
Q (L/hr)	240	28.1	ND
V3 (L)	12.3	11.3	ND
ALAG1 (hr)	0.23	ND	ND
CL (L/hr)	4.71	10.4	27
k_23 (hr^{-1})	7.79	30.3	ND
k_32 (hr^{-1})	19.6	22.8	ND
LBM COV	0.665	11.6	ND
α	0.678	6.38	ND

Parameter uncertainties and inter-individual variability (IIV) are provided when applicable.
ND, not determined; RSE, relative standard error.

FIGURE 1 **PK model simulation of caffeine saliva concentration using data from adolescents study (dots) and previously published studies (straight continuous lines:[15,46,49,50]).** The final measurement time in the adult study is indicated by the vertical dashed line; the assay LOQ is indicated by horizontal dashed line.

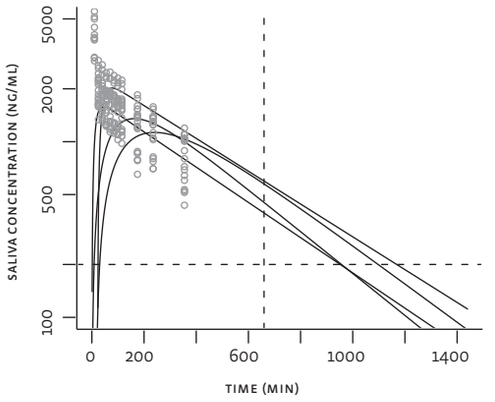


FIGURE 2 **Saccadic Peak Velocity change in least-square mean from baseline profile with 95% CI as error bars.** Rhombus: placebo; circles: caffeine.

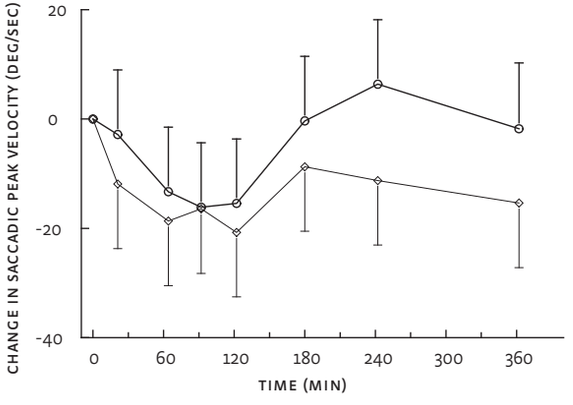


FIGURE 3 Adaptive Tracking Performance change in least-square mean from baseline profile with 95% CI as error bars. Rhombus: placebo; circles: caffeine.

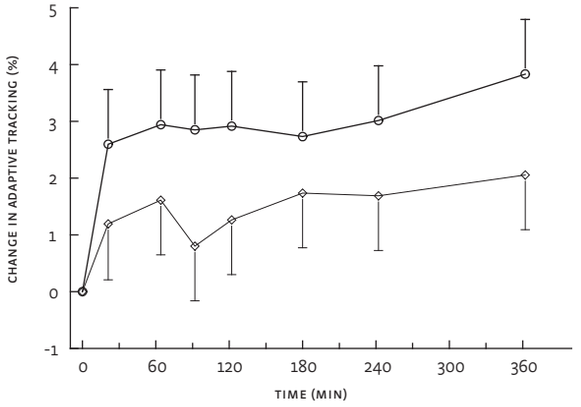


FIGURE 4 Switch Costs error rate (#3) change in least-square mean from baseline profile with 95% CI as error bars. Rhombus: placebo; circles: caffeine.

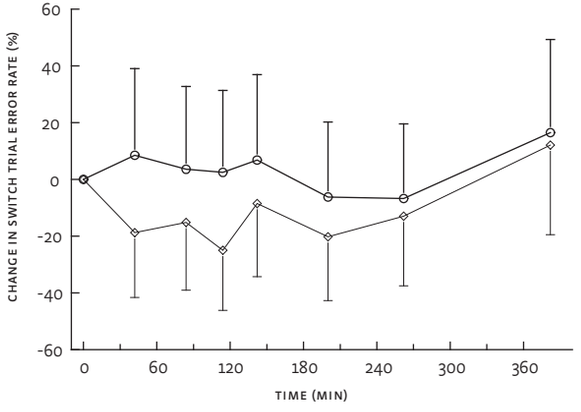


FIGURE 5 Goodness-of-fit plots for the plasma PK model. Upper left: observed (DV) versus population predicted (PRED) plasma caffeine concentrations, using fixed effects only (continuous line represents the line of unity); upper right: observed (DV) versus individual predicted (IPRED), using individual specific empirical Bayes' estimates (continuous line represents the continuous line of unity); lower left: conditional weighted residuals (CWRESI) versus individual predicted (IPRED); lower right: conditional weighted residuals (CWRESI) versus time after dose (TAD). Continuous line represents the Loess fit through the data; horizontal lines represent the mean (0) and +/- 2 standard deviations.

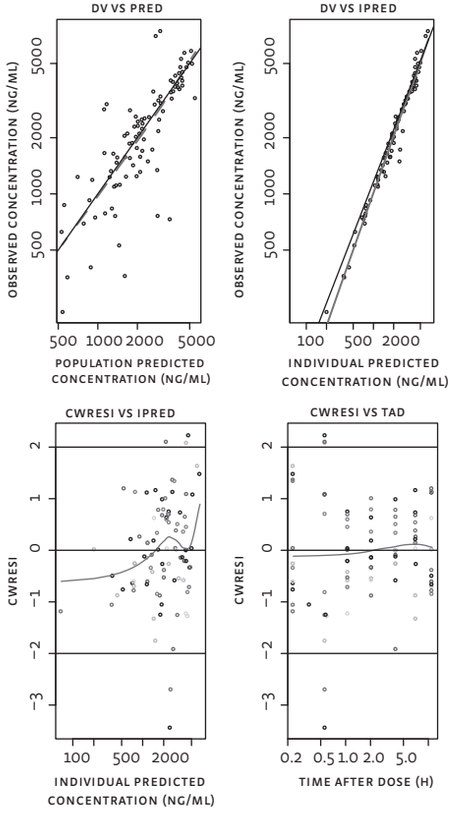


FIGURE 6 Individual caffeine plasma concentration versus time plots on a log-linear scale. Dashed lines represent the population prediction, continuous lines indicate the individual prediction, and circles represent the individual observations. S, subject number; TRT 1, treatment 1 (capsule, 200 mg caffeine); TRT 2, treatment 2 (beverage, estimated dose 90 mg caffeine)

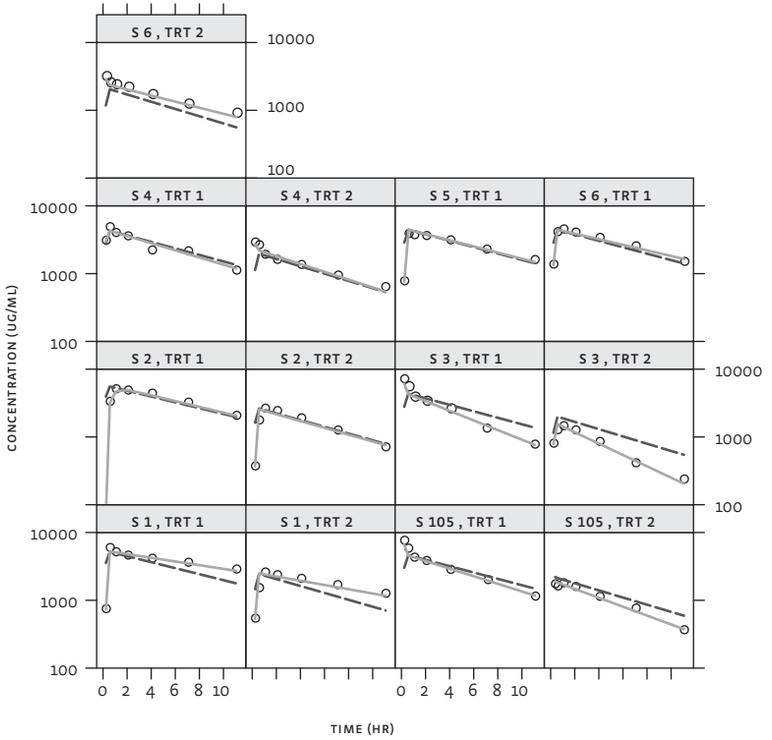


FIGURE 7 Normalized difference between caffeine plasma concentration and corresponding caffeine saliva concentration for the capsule treatment for all subjects. Saliva concentrations were divided by an estimated fraction from the relationship (0.70) in one of the sub-models. Resulting values were then subtracted from the corresponding plasma concentration, then divided by the plasma concentration. This value was then plotted against time after dose.

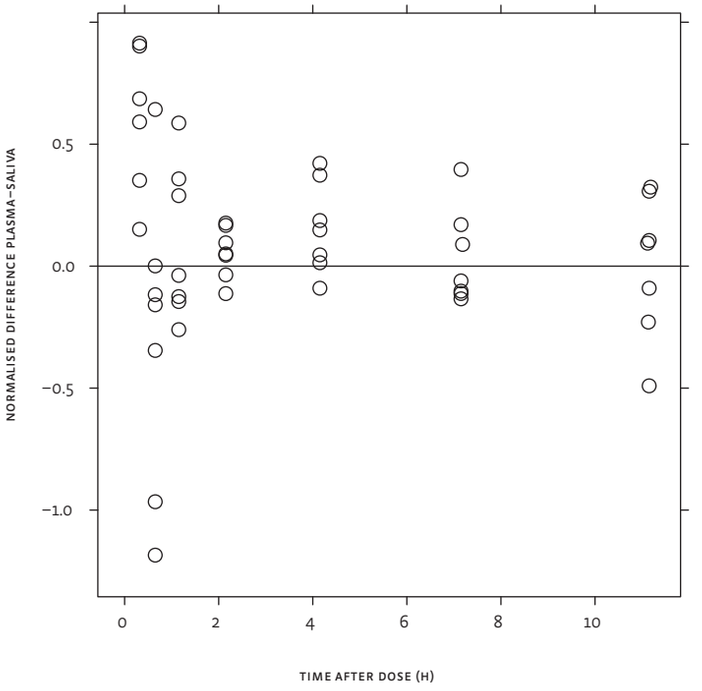


FIGURE 8 **Goodness-of-fit plots for the saliva model.** Upper left: observed (DV) versus population predicted (PRED) saliva caffeine concentrations, using fixed effects only (continuous line represents the line of unity); upper right: observed (DV) versus individual predicted (IPRED), using individual specific empirical Bayes' estimates (continuous line represents the line of unity); lower left: conditional weighted residuals (CWRESI) versus individual predicted (IPRED); lower right: conditional weighted residuals (CWRESI) versus time after dose (TAD). Continuous line represents the Loess fit though the data; horizontal lines represent the mean (0) and +/- 2 standard deviations.

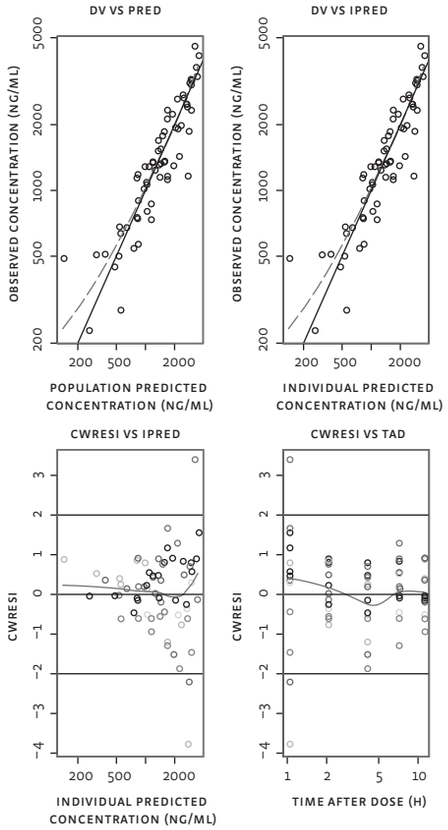


FIGURE 9 **Individual caffeine saliva concentration versus time plots on a log-linear scale.** Continuous lines represent the individual prediction and black circles the observations. Dashed line represent the plasma concentration prediction of the model and grey circles the plasma observations. S, subject number; TRT 1, treatment 1 (capsule, 200 mg caffeine); TRT 2, treatment 2 (beverage, estimated dose 90 mg caffeine)

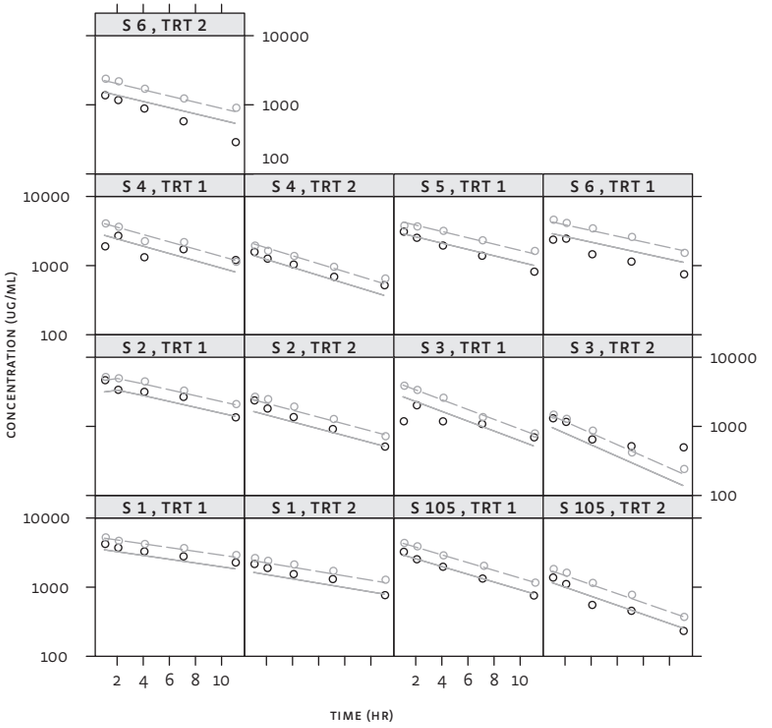
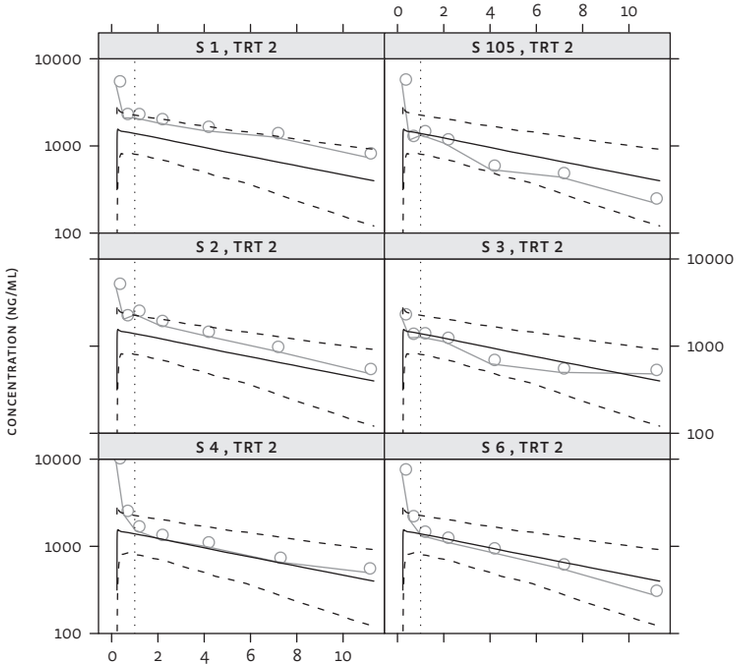
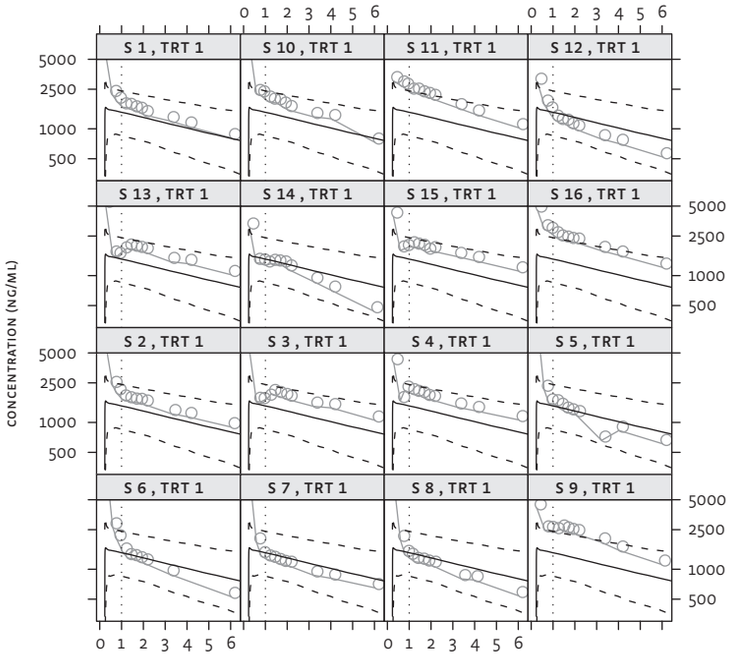


FIGURE 10 Visual predictive check for the saliva concentration after the beverage treatment in (left) adults and (right) adolescents after simulation of 1000 subjects based on the distribution of LBM in the adult and adolescent datasets. Circles represent the observations; black line represents the median prediction; dashed lines represent the 95% prediction interval. Vertical dotted line represents the 1h time point after dose; the saliva model was only based on data from this time point onwards. S, subject number; TRT 1, treatment 1 and TRT 2, treatment 2 (beverage, estimated dose 90 mg caffeine).





CHAPTER 7

Comparison of the pharmacokinetics and effects of alcohol on objective and subjective biomarkers between healthy adolescents and adults

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ABSTRACT

Although the acute effects of alcohol consumption on the central nervous system (CNS) have been studied extensively in adults, these effects have not been studied in adolescents. It is likely that the effects of alcohol reported in adults cannot simply be extrapolated to adolescents, as animal studies have shown that adolescent and adult animals have different sensitivities to alcohol. Here, we used a pharmacokinetics-pharmacodynamics (PK/PD) modeling approach to compare the objective and subjective responses to alcohol between adolescent and adult subjects. The acute effect of consuming a socially accepted dose of alcohol (two standard units) was determined in 16-18-year-old adolescents. Blood alcohol concentration was measured non-invasively using end-expired breath samples. A PK/PD model was then developed by combining the data obtained from this study in adolescents with data obtained from previous alcohol studies performed in adults. This model was used to characterize alcohol's pharmacokinetics and effects on an objective biomarker and a subjective biomarker and to explore potential sources of variability, including age. A two-compartment structural model with first-order absorption and Michaelis-Menten elimination provided the best description of estimated plasma alcohol PK.

Inter-individual variability was identified for several kinetics parameters, with lean body weight-dependent variability in peripheral compartment volume and maximum elimination, weight-dependent variability in central compartment volume, and height-dependent and age-dependent variability in intercompartment clearance. The relationship between alcohol concentration and the effect on baseline smooth pursuit performance and the Visual Analogue Scale (VAS) Alertness score was described best as a dose-dependent effect without indications of delay or tolerance. Higher baseline performance in smooth pursuit was correlated with a larger absolute decrease in performance. No covariates were identified for the relationship between alcohol concentration and effect with respect to baseline smooth pursuit performance or VAS Alertness score. Whether sensitivity to other alcohol-related pharmacodynamics effects changes with age remains to be determined.

Introduction

Ethyl alcohol (ethanol; referred to hereafter as simply 'alcohol') is the most commonly used recreational compound among adolescents[1-3]. In most Western countries, adolescents experiment with alcohol, and alcohol consumption usually becomes 'normal' during adolescence. In 2009, approximately 85% of 15-16-year-old Dutch adolescents reported having consumed alcohol, and more than 60% were current users[2,4]. Concerns regarding the deleterious effects of early or excessive alcohol consumption on brain development and the increased risk of alcohol abuse in adulthood has led to a plethora of experimental animal studies and observational human studies of adolescents with alcohol abuse/dependency or binge drinking (for reviews, see[5-7]). However, although a small subpopulation of adolescents use alcohol with high frequency and are exposed to the risks of heavy drinking, most adolescents ultimately establish a drinking pattern that is considered socially acceptable[2,8-10]. Although adolescents cannot legally purchase alcohol in the Netherlands, alcohol consumption by adolescents is – in itself – not prohibited by Dutch law. Nevertheless, the social and legal acceptability of moderate underage alcohol consumption has no scientific basis, as the functional effects of alcohol in adolescents have not been investigated in a placebo-controlled study.

The effects of acute alcohol consumption on the central nervous system (CNS) have been quantified extensively in adults, and consistent effects on tests that evaluate divided attention and visuomotor control have been reported after a relatively low dose (e.g., BAC <0.5 g/L)[11]. Adolescence is a period of intense development, and animal studies have revealed differences in sensitivity to the acute effects of alcohol between adolescents and adults[12-26]. Therefore, it is conceivable that the effects of alcohol that have been reported in adults cannot simply be extrapolated to adolescents. If human adolescents and adults also have differential sensitivities to the acute effects of alcohol, adolescents may experience functional cognitive impairments at lower alcohol doses than adults, which is a serious issue given the substantial cognitive demands that adolescents face in school and early in their developing careers.

In addition, because motor impairment and sedation are direct effects of moderate alcohol intake[14], lower sensitivity to these effects in adolescence may contribute to increased alcohol use among this age group[27]. Conversely, if adolescents develop severe ataxia or sedation after only a relatively low dose of alcohol, this could impair their ability to ride a bike or drive a car safely. Thus, it is important to determine whether ingesting a 'socially acceptable' quantity of alcohol affects the nervous system with a different time course in adolescents than in adults.

Here, we studied the effect profile of consuming two standard alcohol units by 16-18-year-old adolescent subjects. The acute effects of alcohol were determined using a limited number of well-characterized, sensitive biomarkers[11], and the effects of alcohol on the autonomic nervous system were assessed by measuring systemic blood pressure and heart rate. To determine the correlation between the measured effects and alcohol concentration, blood alcohol concentration was measured non-invasively using end-expired breath samples. A pharmacokinetics-pharmacodynamics (PK/PD) model was developed by combining the data obtained from this study in adolescents with data obtained from alcohol studies in adults performed previously by our research group[28-35]. The purpose of this model was to characterize alcohol pharmacokinetics and alcohol's effects on one objective biomarker and one subjective biomarker and to explore potential sources of variability, including age. Objective and subjective biomarkers for PK/PD modeling were selected based on an exploratory meta-analysis of all relevant alcohol data.

Methods

Clinical trial in healthy adolescents

SUBJECTS

Healthy male and female subjects aged 16-18 years were included. The subjects had to be non smokers. They had to be current users of alcohol (i.e., use of at

least 4 units during the month preceding study participation), but were not allowed to use on average more than 7 units alcohol per week. After signing informed consent (in case age < 18 years, also by parents or legal guardian), subjects were medically screened within three weeks prior to study participation and excluded in case relevant clinical abnormalities were found. Use of medications and compounds known to affect CNS performance (including nicotine, xanthines, drugs or alcohol) were not allowed and were screened during screening and prior to each study day. Ethical approval of the study protocol was obtained from the Central Committee on Research involving Human Subjects, the Netherlands.

STUDY DESIGN

This was a randomized, double-blind, placebo-controlled, two-way crossover study of 16 healthy, adolescent subjects with a wash-out period of at least 3 days. Prior to the study days, subjects were instructed to remain fasted from midnight. Smoking and the use of alcohol and xanthine-containing foods or beverages were not allowed during the study days. A standardised light breakfast and lunch were offered at approximately 1 hr prior and 2.5 hr after alcohol intake respectively. Water was allowed ad libitum. Subjects remained in house until 5 hr after alcohol intake.

The sample size of 16 was calculated (two-sided test, alpha = 0.05) for randomization using Williams Squares. This sample size was estimated to have 80% power of detecting a difference in means of -4.6 points for smooth pursuit eye movement assuming a standard deviation of differences of 6.1 (as found in a previous ethanol study by our group in which an effect of -3.9 points was found for an ethanol level of 0.3 g/L).

INTERVENTIONS

All subjects received 2 beverages (200 ml each) containing an oral dose of approximately 10 gram ethanol each (e.g., appropriate amounts of Malibu Coconut Rum, a rum with natural coconut extract with an alcohol percentage by volume of 21.0%, mixed with coconut milk and orange juice) or placebo (e.g., coconut milk mixed with orange juice and vanilla aroma) on different study days. The total alcohol dose of 20 gram was anticipated to lead to a peak blood concentration of 0.3 g/L. Intervention arms were made as comparable as possible regarding expectancy, sensory effects and the presence of biologically active substances other than ethanol. To avoid confounds from circadian variability, alcohol was administered at the same time of day in all subjects. Subjects were instructed to drink one beverage at a time during a 5 minute interval.

PHARMACOKINETICS

Breath alcohol concentrations (BrAC) were determined through a breath test, using a hand-held Alco-Sensor IV meter (Honac, Apeldoorn, The Netherlands). Subjects were instructed how to properly use the device during a training session and 2 breath tests were taken prior to alcohol or placebo intake. To eliminate residual alcohol in the mouth, subjects were instructed to rinse their mouths thoroughly with water directly after alcohol intake. The first breath test after intake was taken at 17 minutes after alcohol intake to avoid residual alcohol contamination of the mouth despite mouth washing. Breath tests were taken every 20 minutes (until 1 hour after intake), every 30 minutes (until 2 hours after intake) and then at hourly intervals (until 5 hours after intake).

PHARMACODYNAMICS

The NeuroCart is a test battery of sensitive tests for a wide range of CNS domains that has been developed at the Centre for Human Drug Research (CHDR, Leiden, The Netherlands) to examine different types of CNS-active drugs. This test battery was incorporated to provide background information on general CNS performance and functional CNS domains, which could be affected by alcohol based on previous findings in adults[11].
All pharmacodynamic measurements (PD) measurements, with exception of the visual verbal learning task (VVLT), were performed at t= 30, 60, 90, 120, 180, and 300 minutes after alcohol intake. The VVLT was administered at approximately 70 and 140 minutes after alcohol intake to assess immediate and

delayed recall. The PD measurements were performed in a quiet room with ambient illumination with only one subject in the room per session. Prior to the first study day, subjects were familiarized with the experimental procedure and given a practice session on the tasks to minimize learning effects during study days. The tests were performed as described below. In addition, a short questionnaire was taken at screening to evaluate the subjects' current perceptions on alcohol use.

ADAPTIVE TRACKING The adaptive tracking test as developed by Hobbs & Strutt, according to specifications of Borland and Nicholson[36] was used. The adaptive tracking test is a pursuit-tracking task in which a circle of known dimensions moves randomly about a screen. The study subject was instructed to try to keep a dot inside the moving circle by operating a joystick. If this effort was successful, the speed of the moving circle was increased. Conversely, the velocity was reduced if the subject could not maintain the dot inside the circle. Performance was scored after a fixed period and the average performance and the standard deviation of scores over a 3.5-minute period was used for analysis.

SACCADIC EYE MOVEMENTS Saccadic peak velocity is one of the most sensitive parameters for sedation[37–39]. The use of a computer for measurement of saccadic eye movements was originally described by Baloh and colleagues[40], and has been validated at CHDR by Van Steveninck and colleagues[39,41].
Recording and analysis of saccadic eye movements was conducted with a microcomputer-based system for sampling and analysis of eye movements. The nystagmo stimulator used for stimulus display is from Nihon Kohden (Nihon Kohden Corporation, Tokyo, Japan), the program for signal collection and the AD-converter from Cambridge Electronic Design (CED Ltd., Cambridge, UK), the amplification by Grass (Grass-Telefactor, An Astro-Med, Inc. Product Group, Braintree, USA) and the sampling and analysis scripts are developed at the CHDR. Disposable electrodes were applied on the forehead and beside the lateral canthi of both eyes of the subject for registration of the electro-oculographic signals. Head movements were minimised with the aid of a head support placed opposite the target. The target consisted of an array of light emitting diodes on a bar, fixed at 50 cm in front of the head support. Saccadic eye movements were recorded for stimulus amplitudes of approximately 15 degrees to either side. Fifteen saccades were recorded with interstimulus intervals varying randomly between 3 and 6 seconds. Average values of latency (reaction time), saccadic peak velocity of all correct saccades and inaccuracy of all saccades were used as parameters. Saccadic inaccuracy was calculated as the absolute value of the difference between the stimulus angle and the corresponding saccade, expressed as a percentage of the stimulus angle.

SMOOTH EYE PURSUIT The same system as used for saccadic eye movements was also used for measurement of smooth pursuit. For smooth pursuit eye movements, the target moves sinusoidally at frequencies ranging from 0.3 to 1.1 Hz, by steps of 0.1 Hz. The amplitude of target displacement corresponds to 22.5 degrees eyeball rotation to both sides. Four cycles are recorded for each stimulus frequency. The method has been validated at the CHDR by Van Steveninck and colleagues[39,41] based on the work of Bittencourt and colleagues[42] and the original description of Baloh and colleagues[40]. The time in which the eyes were in smooth pursuit of the target was calculated for each frequency and expressed as a percentage of stimulus duration. The average percentage of smooth pursuit for all stimulus frequencies was used as parameter.

DUAL TASK TEST The Dual Task (DT) can be used to measure mental workload, as multiple tasks should produce interference when they compete for the limited capacity resources. During this task, subjects were required to perform two separate tasks, each involving an unrelated mapping of a set of possible stimuli to a set of possible responses[43] in an adapted version of the Pashler's DT[44–46]. This adapted DT consists of 54 trials, with three blocks of 18 trials each. The subject was required to give a response as quickly and as accurately as possible indicating whether the tone is either low or high pitched and which letter is presented with a line below or above it. The subjects were instructed not to postpone their reaction for Stage 1 (S1) up until they knew the response

for Stage 2 (S2). At the end of each block, the subject was provided with feedback of percentage correct responses and mean correct RT for both S1 and S2.

BODY SWAY The body sway meter allows measurement of body movements in a single plane, providing a measure of postural stability and was measured with an apparatus similar to the Wright ataxiameter[37,47]. With a string attached to the waist of the subject, all body movements over a period of time were integrated and expressed as mm sway on a digital display. Before starting a measurement, the subjects were asked to stand still and comfortable, with their feet approximately 10 cm apart and their hands in a relaxed position alongside the body. The subjects were instructed to keep the eyes closed.

VISUAL VERBAL LEARNING TASK The visual verbal learning test (VVLT) is a comprehensive memory task for immediate and delayed recall and contains three different subtests that cover merely the whole scope of learning behaviour. Subjects were asked to complete a training version of the VVLT within three weeks before study start. The subjects were presented 30 words in three consecutive word trials at approximately 75 minutes after alcohol/placebo administration, i.e. word learning test (VVLT30). Each trial ended with a free recall of the presented words, i.e., immediate recall, to determine acquisition and consolidation of information. Approximately two hours after start of the first trial, the subjects were asked to recall as many words as possible, i.e. delayed recall, to measure active retrieval from long term memory. Immediately thereafter, the subjects underwent a memory recognition test, which consisted of 15 presented words and 15 'distractors', i.e. delayed recognition, to test memory storage. The subjects were not allowed to write words down at any time during the whole test procedure.

VISUAL ANALOGUE SCORES At various times, the subject indicated (with a mouse click on the computer screen) on sixteen horizontal Visual Analogue Scales how he/she felt. From these measurements, three main factors were the calculated as described by Bond and Lader[48]: alertness (from nine scores), contentedness (often called mood; from five scores), and calmness (from two scores). To make the VAS more age-appropriate, the words 'incompetent' and 'recalcitrant' in the frequently used Dutch translation of this VAS were replaced by 'onbekwaam' and 'tegendraads' respectively. In addition to this, a separate 100 mm-line was added, asking the subject to indicate 'how large is the effect of alcohol that you feel' (alcohol effect)?

BLOOD PRESSURE AND HEART RATE Automated oscillometric blood pressures were measured using a Nihon-Kohden BSM-1101K monitor, a Colin Pressmate BP 8800 or a Dash 4000 monitor. Pulse rates were determined by oscillometry.

Alcohol studies in adults

Pharmacokinetic and selected pharmacodynamic data from several alcohol studies in adults previously performed by our research group were included in the PK/PD model (see Table 1 for an overview). In these studies, the alcohol clamping method (according to the methods of Zoethout and colleagues[49]) was used in which the infusion rate is adjusted according to the estimated blood alcohol concentration. All studies were performed in healthy adults, with exception of one study in adult patients with essential tremor[30]. In all studies, subjects with a history of ethanol abuse were excluded from the study. In most studies subjects who drank regularly (up to 3-4 alcoholic beverages a day) were allowed to participate. In three studies[28,29,31] familiarity with the use and effects of ethanol was required for inclusion of a subject. All subjects had a normal body mass index (BMI).

Statistical analysis and pharmacometrics

PHARMACODYNAMICS

The pharmacodynamic end-points of the adolescent study were analysed by mixed-model analyses of variance (using SAS PROC MIXED) with treatment,

study day, time and treatment by time as fixed effects, and subject, subject by treatment and subject by time as random effects and the average baseline value as covariate. Contrasts were estimated within the overall treatment effect and contrasts between treatments over 120 min and 300 min were calculated within the statistical model. Body Sway and VAS alcohol scores variables were analysed after log-transformation and back-transformed after analysis (results may be interpreted as percentage change). VAS alcohol scores were log transformed (10log) after 2 was added to each score to avoid log transformation from zero. VVLT parameters were analyzed by mixed model analysis of variance with treatment and study days as fixed factors and subject as random factor. The statistical hypothesis was 'there is no difference between alcohol and placebo'.

PHARMACOMETRICS

DATA PK data from study days in which only alcohol was administered of the adolescent study and several adult studies (see Table 1) were used. Exploratory individual and summary concentration-time profiles were generated to identify potential outliers, understand the influence of censoring concentrations below the limit of detection (BLOD) and give indications regarding the base structural model. As the device reports a value of zero when alcohol breath concentrations are BLOD, all PK observations with a value of zero after plasma peak concentrations were removed from the PK dataset. Blood alcohol concentrations were calculated according to the specifications of the manufacturer (using ratio (2300:1) between blood- and breath ethanol concentrations) and used to develop the PK model.

MODELING STRATEGY Pharmacometric analyses were performed using nonlinear mixed effect modeling (NONMEM version 7.2.0 (Beal). First order conditional estimation with interaction (FOCEI) was used for estimation with a convergence criterion of 5 significant digits in the parameter estimations. NONMEM reports an objective function value (OFV) which is the -2 times log likelihood (-2LL). Model comparison testing was done using the likelihood ratio test under the assumption that the difference in -2LL is Chi-square distributed with degrees of freedom determined by the number of additional parameters in the more complex model. Hence, with a decrease in OFV of at least 10.8 points the model with one additional parameter is considered superior over its parent model ($p<0.001$). Different models with increasing complexity were compared to find the simplest model that described the data adequately. Graphical analysis was used to assess model performance during model development. The goodness of fit plots used included: observed concentration (dependent variable, DV) versus population predicted concentration (PRED) and versus individual predicted concentration (IPRED); weighted residuals (CWRESI) versus IPRED and versus time; combined PRED, IPRED and DV versus time, per individual, and distribution of interindividual variability (ETA). Covariate analysis was performed using a stepwise approach. Selection of the best PK- and best PK/PD models was based on the likelihood ratio test, diagnostic plots, visual predictive check (VPC) and precision in parameter estimates. Calculation of the relative standard error (RSE) was used to derive the uncertainty in the parameter estimates and was considered acceptable when less than 10%. NONMEM input file preparation and processing (tables and graphs) of the model results was performed using R version 2.12.0 (V2.12.0, R Foundation for Statistical Computing, Vienna, Austria, 2010).

POPULATION PK MODEL DEVELOPMENT First, a population PK model for oral and intravenous alcohol was developed. One and two compartment structural models, and different compartmental and elimination submodels were tested. All models used a first order process to describe the oral absorption of alcohol in the adolescent data set. Inter-individual variability (IIV) was assessed separately on each of the PK parameters using a stepwise bottom-up approach. Correlations between the IIV of the various parameters were graphically explored. When correlations were significant, either by shape or Piersons correlation coefficient, covariance between the terms was assessed by application of an omega block on selected parameters and accepted based on the likelihood ratio test. For the parameter estimation, shrinkage was considered

acceptable when below 30%[50]. Proportional, additive, and combined error structures were evaluated to best describe the residual error. After graphical identification, the most promising covariates were tested in the model and included based on decrease in OFV in a stepwise manner (forward inclusion of covariates, followed by a backward elimination step). All covariates were implemented in normalized power function (Eq. 1), where the normalization values for LBW, WGT, AGE and HGT were 60, 70, 30 and 1.75 respectively.

$$\theta i = \exp(\theta TV + \varepsilon) \times ([COV]i / [COV]n) \wedge (\theta cov) \quad \text{Eq. 1}$$

where θi; individual parameter estimate, θTV; typical (population) value, ε; interindividual variability, *COVi*; individual covariate value, *COVn*; normalization value for covariate, θCOV; parameter estimate for the exponent.

PK/PD MODEL DEVELOPMENT After development of the PK model, exploratory PD and PK/PD profiles were generated to identify the most suitable objective- and subjective PD parameter for development of a PK/PD model. Only PD parameters that were measured in at least two different studies and more than once on a single occasion were included. Selection of PD parameters for PK/PD model development was based on strong response to alcohol and indications of presence of a direct relationship between alcohol concentration and effect. For the development of the PK/PD model, all PK parameters were fixed to the individual parameter estimates of the best PK model. The simulated individual plasma alcohol concentration time profiles were used in the exploration of linear- and exponential concentration-effect relationships, to best describe the observed PD response. Model development was performed as described for the best PK model (e.g. incorporation of IIV, residual error and covariates). Model description of the data was considered acceptable if time-, concentration- or performance-dependent bias seemed absent.

Results

Clinical trial in healthy adolescents

SUBJECTS

In the adolescent study, a total of 17 subjects (8 males and 9 females) 16-18 years of age were enrolled in 2010 and 2011. All of the subjects attended pre-university secondary education (vwo, Voortgezet Wetenschappelijk Onderwijs). One of the female subjects discontinued the study because of a migraine experienced following the first study day (an alcohol occasion); this subject was replaced by another female subject. Three of the female subjects used an oral contraceptive; other medications used during the study included levocetirizine 5 mg p.o. as needed (by one subject; stopped two days before the first study occasion), paracetamol 500 mg p.o. 2-6 times daily (by 1 subject during the first study occasion), terbinafine cream 10 mg/g and terbinafine 250 mg p.o. once daily (by one subject during both study occasions). The mean alcohol consumption by the subjects in the months preceding the study was 3 standard units per week (range: 1-6 units per week). The reasons cited by the subjects for drinking alcohol included: they liked it (n=13); 'it is socially enjoyable' (n=14); and it reduces inhibition (n=2). None of the subjects reported that they drank alcohol because their friends also drank alcohol or to become drunk. The subjects either had never been drunk (n=6) or were drunk only occasionally (n=11); nine subjects reported an occasional black-out episode after consuming alcohol. Most of the subjects considered the short-term effects of alcohol (n=7) or the dangers of alcohol consumption (n=11). The majority of subjects (n=11) reported that they considered the long-term effects of alcohol only when pointed out by others.

The mean alcohol dose in the study was 0.29 g/kg (range: 0.24-0.34 g/kg) for males and 0.31 g/kg (range: 0.27-0.38 g/kg) for females. After ingesting the alcohol or placebo on the study day, the subjects were asked which of the two they thought they had received. After ingesting the placebo, 13/16 subjects believed they had taken the placebo, and the other three did not know whether they

had taken placebo or alcohol. After alcohol intake, 14/17 subjects believed they had taken alcohol, two believed they had taken the placebo, and one did not know.

PHARMACODYNAMICS RESULTS

The pharmacodynamics results are summarized in Table 2. Least Square Means graphs of the parameters that differed significantly between alcohol and placebo are presented in the Supplemental Data.

PHARMACOMETRICS

An overview of the demographics for the studies from which data were used to develop the model is presented in Table 3.

PK MODEL DEVELOPMENT A total of 3,112 PK observations were obtained from 27 adolescents (16-18 years of age) and 122 adult subjects (19 years of age and older). In general, the percentage of PK data that was below the level of detection was <15%, except for the adult study in healthy Caucasian and Japanese volunteers[29] (34%, excluding pre-dose observations) and the adult study with the compound GSK598809[32] (29%, excluding pre-dose observations). In these two studies, PK sampling continued relatively long after ethanol infusion had stopped, and during the last few PK measurements, most of the subjects' alcohol concentrations were below detectable levels. None of the data points between the start of dosing and reaching peak plasma alcohol concentration were below detectable levels. The entire data set from one occasion from one adult subject was excluded because the subject was not 'well-rested', and the occasion was repeated on a later day. Finally, data from four adult subjects were excluded due to the suspected presence of erroneous dosing information.

A two-compartment structural model with first-order absorption and Michaelis-Menten elimination provided the best description of estimated alcohol plasma PK. A schematic representation of the alcohol PK model structure is presented in Figure 3. Parameter estimates, relative standard error (RSE), and IIV are presented in Table 4. A combined residual error structure was identified for the model. IIV could be identified with respect to VM (the maximum elimination rate parameter), V2 (the central compartment), V3 (the volume of the peripheral compartment), and Q (intercompartment clearance). The subsequent addition of an ETA to the Michaelis constant (KM) caused a significant decrease in OFV; however, this decrease (38%) was above the acceptance criterion and this covariate was not incorporated in the model. The investigated covariates included age, gender, height, weight, BMI, and lean body weight (LBW[51]). The following five covariate relationships were implemented in the model: LBW on V3 and VM, weight on V2, and height on Q, and age on Q. For the covariate weight on V2, the exponent was fixed at 1, as the confidence interval of the estimated exponent overlapped 1.0. Backwards elimination was than performed; however, this approach resulted in a significantly poorer model in all cases. Covariance was identified between VM and Q, VM and V2, and V2 and V3.

In the best PK model, the predicted concentrations were accurate and no time-dependent bias was observed (Figure 4 and 5). The Loess regression curve of the conditional weighted residuals (CWRESI) versus individual predicted concentration (Figure 5) suggested a slight bias at higher concentrations (>1.0 g/L); this bias is likely due to the low number of observations at high concentrations (there were only two observations between 1.5 and 2.0 g/L) reducing the reliability of the Loess curve, rather than to model misspecification. NONMEM assumes that ETAs are normally distributed around zero. The estimated ETAs were distributed normally (Figure 6).

Because alcohol was administered orally in only one study (adolescent study), and because this study did not include crossover design with an intravenous group, oral bioavailability (F) could not be estimated reliably by the PK model. The individual parameter estimates of V2, V3, and VM of the subjects in the adolescent study actually represent V2/F, V3/F, and VM /F, which can affect parameter estimation and/or the best model structure. A sensitivity analysis was performed using the data set (excluding the oral data). Removal of the oral data from the analysis did not change any decisions made at key steps in the model development; the resulting best model structure was the same, and

none of the parameter estimates changed significantly. Therefore, we conclude that including oral data – without estimating oral bioavailability – did not have a negative impact on the model development.

POPULATION PK/PD MODEL DEVELOPMENT Based on the exploratory plots of PD and PK/PD, smooth pursuit performance was chosen as the most suitable objective PD measure (3643 data points collected from 24 adolescent subjects and 64 adult subjects), and VAS Alertness (2671 data points collected from 19 adolescent subjects and 68 adult subjects) was chosen as the most suitable subjective PD measure. For these parameters, the exploratory plots suggested a strong, dose-dependent alcohol effect with no placebo effect (Figures 1 and 2). The exploratory plots also suggested relatively large inter-individual differences in subject susceptibility to alcohol's effects on smooth pursuit performance and VAS Alertness.

Smooth pursuit was included as a PD measure in the adolescent study and in four of the adult studies[28,29,32,35], and VAS Alertness was included as a PD measure in the adolescent study and in four of the adult studies[29,31,32,35]. One of the adolescent pre-dose smooth pursuit measurements reflected extremely poor performance (5.2%), which was likely related to a technical or subject attention-related problem; this measurement was therefore excluded from the analysis.

SMOOTH PURSUIT PK/PD MODEL The relationship between alcohol concentration and the effect on baseline smooth pursuit performance was described best as a direct, linear concentration-effect relationship. A concentration-effect relationship proportional to the baseline characterized the effects of ethanol on smooth pursuit performance better than an absolute effect independent of baseline (lower OFV and comparable parameter estimates). Because our exploratory plots revealed no apparent placebo effect on smooth pursuit performance, we used a fixed baseline. We also attempted to estimate a population value for the baseline performance; however, the best results were obtained using the mean performance of the pre-dose smooth pursuit tests as the baseline. Implementing an additional population value did not significantly improve the model ($p>0.05$).
The data were described using the following equation:

$$SMP = BL \times (1-(KE \times C)) \qquad \text{Eq. 2}$$

where SMP is smooth pursuit performance (%), BL is baseline performance (%), KE is effect constant (L/g), and C (g/L) is the alcohol concentration simulated by the plasma PK model.

An additive error structure performed better than a proportional error structure in all models (including the best PK/PD model). A combined error structure did not improve the model and was abandoned. IIV could be identified for the baseline smooth pursuit performance (BL) and the effect constant (KE), with acceptable shrinkage in the ETA on BL and KE (<20%). A covariate analysis of age and gender was performed. Including age as a continuous covariate did not improve model performance. Age was also implemented as a categorical covariate, parsing the subjects into the following three groups: adolescents (16-18 years of age), young adults (19-29 years of age), and older adults (>29 years of age). This approach did not reduce OFV, and the estimated age effect did not differ significantly from 'no effect'. As a result, no covariate was included on the model parameters BL and KE. Implementation of covariance between ETA1 and ETA2 did not improve the model and was therefore not added to the model.

The parameter estimates of the best model are summarized in Table 5. The uncertainty of the parameter estimates was considered to be acceptable. As expected based on the exploratory plots, the IIV of KE was relatively high (60.7%). Both the shrinkage on the ETAs and the additive error were below 20% and were therefore considered to be acceptable.

The ability of the PK/PD model to describe the effect of alcohol on smooth pursuit performance was acceptable (Figures 7 and 8). NONMEM assumes a normal distribution of the ETAs around zero. In the best model, the ETAs follow this assumption, although the distribution of the ETA1 suggests a slight skew (Figure 9).

Because the PK/PD model was developed by sequentially modeling PK and PD, mis-fitting of the PK can cause a bias in the predicted PD (for example, if the ethanol concentrations are overestimated, the predicted effect might be overestimated as well). Therefore, the conditional weighted residuals of the best PK model were plotted against the residuals of the best PK/PD model. We found no indication that the residual error in the best PK/PD model was caused by the residual error in the best PK model (Figure 10).

VAS Alertness PK/PD model

The relationship between alcohol concentration and VAS Alertness score was described best as a direct, linear effect on baseline. An additional concentration-effect relationship characterized the effects of ethanol on VAS Alertness best when compared to a proportional relationship. Because there were no indications of a placebo effect, a fixed baseline was chosen. We tested the following four baseline sub-models: (1) the estimated population value; (2) the occasion-specific mean of the pre-dose measurements; (3) the fraction of the occasion-specific mean of the pre-dose measurements, and (4) the occasion-specific mean of the pre-dose measurements as a normalized, linear covariate on the estimated population value. Of these four sub-models, the fourth performed the best ($p<0.001$) and was therefore implemented in the structural model. A combined error structure provided the best characterization of the residual error in the best model.
The data were described using to the following equation:

$$\text{VAS Alert} = BL - (KE \times C) \qquad \text{Eq. 3}$$

where VAS Alert is the VAS Alertness score (mm), BL is the baseline score (mm), KE is the effect constant (mm*g/L), and C (g/L) is the predicted alcohol concentration.

Removing either the additive or proportional error component resulted in a significantly poorer model ($p<0.001$). Implementing IIV on KE significantly improved the model ($p<0.001$), but because of high ETA-shrinkage (42.7%), the model without IIV on BL was deemed superior. The occasion-specific mean of the pre-dose measurements was used as a covariate on BL during the structural model development. Because none of the covariates showed a particularly strong correlation with the individual parameter estimates (based on Pearson's correlation coefficient and a visual check of the scatterplot), no additional covariate relationships were included in the model.

The parameter estimates are presented in Table 6. The uncertainty of the residual error was rather high, and the other parameters had a reasonable degree of uncertainty. As expected from the exploratory plots, the IIV of KE was high compared to the population estimate of KE. Both the shrinkage of ETA and the error were <20%.

The PK/PD model described the effect of ethanol on VAS Alertness score relatively well, with an absence of both time-dependent and concentration-dependent bias (Figures 11 and 12). The Loess curve in the CWRESI vs IPRED plot suggests a bias towards overestimating the VAS Alertness score at very low scores (<30 mm). However, given the relatively small contribution of these few values in the total number of observations and given the fact that the low CWRESI values seemed to lie within acceptance limits (-2 to 2), this apparent bias was not considered a factor in the model's performance. NONMEM assumes a normal distribution of the ETAs around zero. The IIV of KE followed a normal distribution, although the mean of ETA was not significantly different from zero (Figure 13). Thus, it might not be appropriate to assume a normal distribution of ETA when performing a simulation using this PK/PD model.

Discussion

This is the first study to use a PK/PD modeling approach to compare the objective and subjective responses to alcohol between adolescents and adults. Most previous observational studies of alcohol use in adolescent subjects focused on potential negative effects on the brain and the associated long-term

risks of heavy drinking. Only a few clinical studies investigated the effects of acute alcohol in children and adolescents; however, these studies were not placebo-controlled and included relatively alcohol-naïve subjects[52] or they evaluated responses in adolescents who had (or were at high risk for developing) an alcohol abuse disorder[52,53]. Data from these studies may not have direct implications for how alcohol interferes with psychomotor and cognitive abilities in more common situations, for example when relatively low, socially accepted doses are consumed by healthy adolescents with a limited history of drinking alcohol. In our study, a PK/PD model was developed by combining the adolescent data with adult data obtained from alcohol studies previously performed by our research group in order to investigate whether PK and/or PD has any age-related differences. The clamping method, which was used in previous adult studies, is best suited to evaluating potential sources of variance in PK, including age-related effects. Due to ethics considerations, ethanol clamping could not be performed in adolescents. We anticipated that the accuracy of the clamping method in adults would provide the basis for an accurate PK/PD model, which would be robust enough to accommodate the more variable adolescent data collected after oral ingestion.

The PK model was built using a combination of estimated blood alcohol concentration in adolescents (following oral administration) and adults (using an infusion-based clamping method). A two-compartment structural model with first-order absorption and Michaelis-Menten elimination provided the best description of estimated alcohol plasma PK. The estimated combined volume of the central and peripheral compartments (46 liters) and estimates of elimination (maximum elimination rate and Michaelis constant) were consistent with published values[54–57]. The population parameter estimates of absorption and distribution had low uncertainties. Combining oral (low concentration range) and intravenous (higher concentration range) PK data provided an accurate estimate of the absorption rate, distribution parameters, and Michaelis constant, thereby yielding accurate descriptions of the ascending and descending limbs of the alcohol concentration-time curve (the PK phases in which most of alcohol's effects occur). In contrast, the relative standard error of the population Km value in our PK model was rather high. Because the majority of data was collected during clamping experiments, a relatively low percentage of the data was in the low concentration range, thereby reducing the informative value of the data with respect to estimating Km. In the adolescent study, alcohol was given as a fixed dose of 20 mg rather than being adjusted for weight (or total body water, which might have been preferred[58]). While this approach might reflect common drinking practices, it likely added variability. Interindividual variability was identified for various kinetic parameters, with LBW-dependent variability on peripheral compartment volume and maximum elimination, weight-dependent variability on central compartment volume, and height- and age-dependent variability on intercompartment clearance. Body composition is important for the equilibrium distribution of alcohol between the blood and various body compartments[58]. Because alcohol is distributed into the total body water, differences in age, gender, and body weight can affect alcohol's concentration-time profile[59–61]. In our evaluation, gender per se was not identified as a potential covariate, but gender was factored indirectly into in the calculation of LBW. The covariates that were identified in our PK model may be related – either directly or indirectly – to differences between adolescents and adults, as considerable age-related and maturity-related changes in body composition occur during adolescence, including changes in weight, height, and fat-free mass[62]. Because all of our subjects had normal BMI, differences in the prevalence of obesity between the adolescents and adults could not have accounted for the weight-related variability. The rate of alcohol distribution is dependent on factors that govern peripheral distribution[58]. In our PK model, age-dependent variability was found with respect to intercompartment clearance; specifically, clearance increases with age. Changes in intercompartment distribution can be caused by changes in peripheral circulation (e.g., due to stress), muscle contraction, hormonal changes, vasoconstriction, changes in body and environmental temperature, and circulatory impairments in the cardiovascular system[58]. Moreover, it is conceivable that adolescent subjects have different stress reactions to the testing environment than adults.

However, age-dependent factors unrelated to the testing environment may play a role as well, as equilibration can also reflect the development of other age-dependent processes, including alcohol- or metabolite-related changes in local muscle and/or CNS blood flow.

Although many studies have examined the acute effects of alcohol in adults[11], to date no alcohol PK/PD model has been presented. This might be due to the complex PD of alcohol, as both acute tolerance and a lag in the recovery from alcohol-induced impairment have been observed for several biomarkers[65-68], thus complicating the development of a PK/PD model. In our study, two biomarkers were selected based on an exploratory meta-analysis of several alcohol studies that were performed by our research group. Smooth pursuit performance and VAS Alertness showed a clear response to alcohol with no indications of an indirect effect or acute tolerance. Thus, a relatively simple model would likely describe the data well, and the presence or absence of an age-related effect could be investigated. As expected, the relationship between alcohol concentration and the effects of alcohol on baseline smooth pursuit performance and VAS Alertness score was described best as being dose-dependent, with no indications of delay or tolerance. Higher baseline performance for smooth pursuit was correlated with a larger absolute decrease in performance. The best model accounted for 61% and 13% of the inter-individual variability in smooth pursuit performance and VAS Alertness score, respectively, and the best model predicted the observed performance accurately. No significant covariates were identified, including no clear effect of age. However, VAS alertness and smooth pursuit eye movements may not necessarily represent all of the alcohol-related effects on the CNS, and we cannot exclude the possibility that sensitivity to other alcohol-related pharmacodynamics effects change with age. Age-related differences between adolescents and adults with respect to acute sensitivity to alcohol are believed to reflect PD –rather than PK –factors[72], and these differences may be related to the faster onset of acute tolerance and developmental changes that occur in the neural substrates underlying alcohol's effects. For example, age-related differences in sensitivity and tolerance have been related to age- and brain region-related differences in the expression NMDA and $GABA_A$ receptor isoforms[14]. In our study, smooth pursuit performance and VAS Alertness score were analyzed, as the data were likely to be described best using a simple PK/PD model. Other sensitive functional biomarkers[11] that were included in the adolescent study (for example, adaptive tracking, saccadic peak velocity, and VAS alcohol effect) were less suitable for developing a PK/PD model, as the small effect in adolescents and the high number of non-responders precluded our ability to quantify the adolescent data and evaluate an age-dependent effect. Although a higher dose of alcohol would likely have yielded a quantifiable effect, using a higher dose in adolescents would have been prohibitive from an ethics perspective. Other age-dependent effects may have be seen at higher doses as well, including effects on memory, given that a placebo-controlled study[73] found that 0.6 g/kg alcohol caused significantly more memory impairment in 21-24-year-old subjects than in 25-29-year-old subjects. In addition, adolescents and adults may also differ with respect to the development of acute tolerance to alcohol effects, as has been reported for animals[14]. Tests that evaluate postural stability (for example, body sway) may also reveal an age-dependent effect and may reflect a difference in the threshold to the aldehyde metabolite rather than the alcohol itself. Age-related differences in the PK and/or PD of acetaldehyde, which can mediate physiological responses such as facial redness, pulse rate, and blood pressure[63], may account for some of the differences observed between adolescents and adults[64], although this topic needs further study.

Research into age-dependent differences in the effects of alcohol is potentially complicated by differences in cumulative baseline drinking (affecting PK and/or PD) and age-related differences in motivation and expectations (affecting PD). In all of the adult studies, alcohol was administrated intravenously using the clamping method, which provides precise control over blood alcohol content (and therefore the brain's exposure to alcohol), thus minimizing variation between subjects. Due to ethics considerations, ethanol clamping was not performed in adolescents. The PK model adequately described both the oral and intravenous PK data. Adult subjects were exposed to alcohol longer than

the adolescents were exposed, although the clamp duration had no impact on VAS Alertness or smooth pursuit eye movement. However, it is formally possible that the route of administration can affect the brain's response to alcohol[87]. For example, the brain's functional response to alcohol can depend on both the alcohol concentration and the rate of change of alcohol concentration[88], which can differ depending on the route of administration.

The clear effects revealed by our study (including effects on motor coordination, subjective alertness and intoxication, postural stability, and vital signs) are an insightful illustration of the acute functional impact that healthy adolescents experience following a low dose of alcohol that is generally considered to be socially acceptable. Based on the clear effects measured in our study, the Dutch upper limit for blood alcohol concentration for drivers up to 23 years of age (0.2 g/L) seems justified and should perhaps be lowered to 0.0 g/L, at least for 18-year-old drivers. Importantly, the risk of auto accidents among adolescents is higher than in adults, particularly at low and moderate blood alcohol concentrations[86]. In our PK model an age-dependent variability was found for intercompartmental clearance of alcohol, which may be related to age-dependent factors related to the testing environment or reflect development of post-synaptic age-dependent processes. No covariates could be identified for the relationship between alcohol concentration and effect on baseline smooth pursuit performance or VAS Alertness score. It remains to be investigated if the sensitivity to other pharmacodynamic effects of alcohol does change with age.

REFERENCES

1 Hibell B, Guttormsson U, Ishröm SB, akireva O, B, rnason T, et al. The 2012 ESPAD report: substance use among students in 36 European countries. The Swedish Council for Information on Alcohol and Other Drug and the Pompidou Group of the Council of Europe,Stockholm . 2012.
2 van Laar. Annual Report NDM, 2010, Netherlands National Drug Monitor, Trimbos Institute, Utrecht.
3 Johnston. Monitoring the Future national results on adolescent drug use: Overview of key findings, 2011. Ann Arbor: Institute for Social Research, The University of Michigan.
4 Monshouwer. Jeugd en Riskant Gedrag 2007. Kerngegevens uit het Peilstationsonderzoek Scholieren [Youth and Risky Behavior 2007. Core Figures from Monitoring Study for Students] Trimbos Institute, Utrecht (2008).
5 Guerri C, Pascual M. Mechanisms involved in the neurotoxic, cognitive, and neurobehavioral effects of alcohol consumption during adolescence. *Alcohol.* 2010;44:15-26.
6 Maldonado-Devincci AM, Badanich KA, Kirstein CL. Alcohol during adolescence selectively alters immediate and long-term behavior and neurochemistry. *Alcohol.* 2010;44:57-66.
7 Witt ED. Research on alcohol and adolescent brain development: opportunities and future directions. *Alcohol.* 2010;44:119-124.
8 Geels LM, Bartels M, van Beijsterveldt TC, Willemsen G, van der Aa N, Boomsma DI, et al. Trends in adolescent alcohol use: effects of age, sex and cohort on prevalence and heritability. *Addiction.* 2012;107:518-527.
9 Bauman A, Phongsavan P. Epidemiology of substance use in adolescence: prevalence, trends and policy implications. *Drug Alcohol Depend.* 1999;55:187-207.
10 Malta DC, Mascarenhas MD, Porto DL, Duarte EA, Sardinha LM, Barreto SM, et al. Prevalence of alcohol and drug consumption among adolescents: data analysis of the National Survey of School Health. *Rev Bras Epidemiol.* 2011;14 Suppl 1:136-146.
11 Zoethout RW, Delgado WL, Ippel AE, Dahan A, van Gerven JM. Functional biomarkers for the acute effects of alcohol on the central nervous system in healthy volunteers. *Br J Clin Pharmacol.* 2011;71:331-350.
12 Varlinskaya EI, Spear LP. Ethanol-induced social facilitation in adolescent rats: role of endogenous activity at mu opioid receptors. *Alcohol Clin Exp Res.* 2009;33:991-1000.
13 Varlinskaya EI, Spear LP. Acute effects of ethanol on social behavior of adolescent and adult rats: role of familiarity of the test situation. *Alcohol Clin Exp Res.* 2002;26:1502-1511.
14 Spear LP, Varlinskaya EI. Adolescence. Alcohol sensitivity, tolerance, and intake. *Recent Dev Alcohol.* 2005;17:143-159.
15 Silveri MM, Spear LP. Acute, rapid, and chronic tolerance during ontogeny: observations when equating ethanol perturbation across age. *Alcohol Clin Exp Res.* 2001;25:1301-1308.
16 White AM, Truesdale MC, Bae JG, Ahmad S, Wilson WA, Best PJ, et al. Differential effects of ethanol on motor coordination in adolescent and adult rats. *Pharmacol Biochem Behav.* 2002;73:673-677.
17 Philpot RM, Badanich KA, Kirstein CL. Place conditioning: age-related changes in the rewarding and aversive effects of alcohol. *Alcohol Clin Exp Res.* 2003;27:593-599.
18 White AM, Swartzwelder HS. Age-related effects of alcohol on memory and memory-related brain function in adolescents and adults. *Recent Dev Alcohol.* 2005;17:161-176.
19 Markwiese BJ, Acheson SK, Levin ED, Wilson WA, Swartzwelder HS. Differential effects of ethanol on memory in adolescent and adult rats. *Alcohol Clin Exp Res.* 1998;22:416-421.
20 Silveri MM, Spear LP. Ontogeny of ethanol elimination and ethanol-induced hypothermia. *Alcohol.* 2000;20:45-53.

21 Swartzwelder HS, Richardson RC, Markwiese-Foerch B, Wilson WA, Little PJ. Developmental differences in the acquisition of tolerance to ethanol. *Alcohol.* 1998;15:311-314.
22 Ristuccia RC, Spear LP. Adolescent ethanol sensitivity: hypothermia and acute tolerance. *Ann N Y Acad Sci.* 2004;1021:445-447.
23 Ristuccia RC, Spear LP. Sensitivity and tolerance to autonomic effects of ethanol in adolescent and adult rats during repeated vapor inhalation sessions. *Alcohol Clin Exp Res.* 2005;29:1809-1820.
24 Hefner K, Holmes A. An investigation of the behavioral actions of ethanol across adolescence in mice. *Psychopharmacology (Berl).* 2007;191:311-322.
25 Quoilin C, Didone V, Tirelli E, Quertemont E. Ontogeny of the stimulant and sedative effects of ethanol in male and female Swiss mice: gradual changes from weaning to adulthood. *Psychopharmacology (Berl).* 2010;212:501-512.
26 Schwandt ML, Barr CS, Suomi SJ, Higley JD. Age-dependent variation in behavior following acute ethanol administration in male and female adolescent rhesus macaques (Macaca mulatta). *Alcohol Clin Exp Res.* 2007;31:228-237.
27 Van Skike CE, Botta P, Chin VS, Tokunaga S, McDaniel JM, Venard J, et al. Behavioral effects of ethanol in cerebellum are age dependent: potential system and molecular mechanisms. *Alcohol Clin Exp Res.* 2010;34:2070-2080.
28 Zoethout RW, Schoemaker RC, Zuurman L, van PH, Dahan A, Cohen AF, et al. Central nervous system effects of alcohol at a pseudo-steady-state concentration using alcohol clamping in healthy volunteers. *Br J Clin Pharmacol.* 2009;68:524-534.
29 Zoethout RW, de Kam ML, Dahan A, Cohen AF, van Gerven JM. A comparison of the central nervous system effects of alcohol at pseudo-steady state in Caucasian and expatriate Japanese healthy male volunteers. *Alcohol.* 2012;46:657-664.
30 Zoethout RW, Iannone R, Bloem BR, Palcza J, Murphy G, Chodakewitz J, et al. The effects of a novel histamine-3 receptor inverse agonist on essential tremor in comparison to stable levels of alcohol. *J Psychopharmacol.* 2012;26:292-302.
31 Khalili-Mahani N, Zoethoet RM, Beckmann CF, Baerencs E, de Kam ML, Soeter RP, et al. Effects of morphine and alcohol on functional brain connectivity during 'resting state': a placebo-controlled crossover study in healthy young men. *Hum Brain Mapp.* 2012;33:1003-18.
32 te Beek ET, Zoethout RW, Bani MS, Andorn A, Iavarone L, Klaassen ES, et al. Pharmacokinetics and central nervous system effects of the novel dopamine D3 receptor antagonist GSK598809 and intravenous alcohol infusion at pseudo-steady state. *J Psychopharmacol.* 2012;26:303-314.
33 te Beek ET, Tatosian D, Majumdar A, Selverian D, Klaassen ES, Petty KJ, et al. Placebo- and amitriptyline-controlled evaluation of central nervous system effects of the NK1 receptor antagonist aprepitant and intravenous alcohol infusion at pseudo-steady state. *J Clin Pharmacol.* 2013;53:846-856.
34 Hoch M, Hay JL, Hoever P, de Kam ML, te Beek ET, van Gerven JM, et al. Dual orexin receptor antagonism by almorexant does not potentiate impairing effects of alcohol in humans. *Eur Neuropsychopharmacol.* 2013;23:107-117.
35 Stockis A, Kruithof AC, van Gerven JMA, de Kam ML, Watanabe S, Peeters PA. Interaction study between brivaracetam and ethanol in healthy subjects. Submitted abstract to AES 2014 . 2014.
36 Borland RG, Nicholson AN. Visual motor co-ordination and dynamic visual acuity. *Br J Clin Pharmacol.* 1984;18 Suppl 1:69S-72S.
37 van Steveninck AL, van Berckel BN, Schoemaker RC, Breimer DD, van Gerven JM, Cohen AF. The sensitivity of pharmacodynamic tests for the central nervous system effects of drugs on the effects of sleep deprivation. *J Psychopharmacol.* 1999;13:10-17.
38 van Steveninck AL, Schoemaker HC, Pieters MS, Kroon R, Breimer DD, Cohen AF. A comparison of the sensitivities of adaptive tracking, eye movement analysis and visual analog lines to the effects of incremental doses of temazepam in healthy volunteers. *Clin Pharmacol Ther.* 1991;50:172-180.
39 van Steveninck AL. Methods of assessment of central nervous system effects of drugs in man. State University Leiden. 1994.
40 Baloh RW, Sills AW, Kumley WE, Honrubia V. Quantitative measurement of saccade amplitude, duration, and velocity. *Neurology.* 1975;25:1065-1070.
41 van Steveninck AL, Cohen AF, Ward T. A microcomputer based system for recording and analysis of smooth pursuit and saccadic eye movements. Br.J.Clin Pharmacol. 27, 712-713. 1989.
42 Bittencourt PR, Wade P, Smith AT, Richens A. Benzodiazepines impair smooth pursuit eye movements. *Br J Clin Pharmacol.* 1983;15:259-262.
43 Pashler H. Dissociations and Dependencies between Speed and Accuracy: Evidence for a Two-Component Theory of Divided Attention in Simple Tasks. Cognitive Psychology 21, 469-515. 1989.
44 Pashler H. Attentional limitations in doing two tasks at the same time. Current Directions in Psychological Science 1, 44-47. 1992.
45 Hazeltine E, Ruthruff E. Modality pairing effects and the response selection bottleneck. *Psychol Res.* 2006;70:504-513.
46 Ruthruff E, Hazeltine E, Remington RW. What causes residual dual-task interference after practice? *Psychol Res.* 2006;70:494-503.
47 Wright BM. A simple mechanical ataxiameter. *J Physiol.* 1971;218:27P-28P.
48 Bond A, Lader M. The use of analogue scales in rating subjective feelings. Br.J.Med. Psychol 47, 211-218. 1974.
49 Zoethout RW, van Gerven JM, Dumont GJ, Paltansing S, van Burgel ND, van der Linden M, et al. A comparative study of two methods for attaining constant alcohol levels. *Br J Clin Pharmacol.* 2008;66:674-681.
50 Savic RM, Karlsson MO. Importance of shrinkage in empirical bayes estimates for diagnostics: problems and solutions. AAPS *J.* 2009;11:558-569.
51 Hume R. Prediction of lean body mass from height and weight. *J Clin Pathol.* 1966;19:389-391.
52 Behar D, Berg CJ, Rapoport JL, Nelson W, Linnoila M, Cohen M, et al. Behavioral and physiological effects of ethanol in high-risk and control children: a pilot study. *Alcohol Clin Exp Res.* 1983;7:404-410.
53 Miranda R, Jr., Monti PM, Ray L, Treloar HR, Reynolds EK, Ramirez J, et al. Characterizing subjective responses to alcohol among adolescent problem drinkers. *J Abnorm Psychol.* 2014;123:117-129.
54 Norberg A, Gabrielsson J, Jones AW, Hahn RG. Within- and between-subject variations in pharmacokinetic parameters of ethanol by analysis of breath, venous blood and urine. *Br J Clin Pharmacol.* 2000;49:399-408.
55 Seng KY, Limenta LM, Heng D, Lee EJ. Population pharmacokinetics and pharmacogenetics of alcohol in Chinese and Indians in Singapore. *J Clin Pharm Ther.* 2013;38:141-149.
56 Shoaf SE. Pharmacokinetics of intravenous alcohol: two compartment, dual Michaelis-Menten elimination. *Alcohol Clin Exp Res.* 2000;24:424-425.
57 Holford NH. Clinical pharmacokinetics of ethanol. *Clin Pharmacokinet.* 1987;13:273-292.
58 Norberg A, Jones AW, Hahn RG, Gabrielsson JL. Role of variability in explaining ethanol pharmacokinetics: research and

forensic applications. *Clin Pharmacokinet.* 2003;42:1-31.
59 Jones AW, Neri A. Age-related differences in blood ethanol parameters and subjective feelings of intoxication in healthy men. *Alcohol Alcohol.* 1985;20:45-52.
60 Devgun MS, Dunbar JA. Alcohol consumption, blood alcohol level and the relevance of body weight in experimental design and analysis. *J Stud Alcohol.* 1990;51:24-28.
61 Davies BT, Bowen CK. Total body water and peak alcohol concentration: a comparative study of young, middle-age, and older females. *Alcohol Clin Exp Res.* 1999;23:969-975.
62 Guo SS, Chumlea WC, Roche AF, Siervogel RM. Age- and maturity-related changes in body composition during adolescence into adulthood: the Fels Longitudinal Study. *Int J Obes Relat Metab Disord.* 1997;21:1167-1175.
63 Bae KY, Kim SW, Shin HY, Kim JM, Shin IS, Kim SJ, et al. The acute effects of ethanol and acetaldehyde on physiological responses after ethanol ingestion in young healthy men with different ALDH2 genotypes. *Clin Toxicol (Phila).* 2012;50:242-249.
64 Cao J, Belluzzi JD, Loughlin SE, Keyler DE, Pentel PR, Leslie FM. Acetaldehyde, a major constituent of tobacco smoke, enhances behavioral, endocrine, and neuronal responses to nicotine in adolescent and adult rats. *Neuropsychopharmacology.* 2007;32:2025-2035.
65 Holford NH. Complex PK/PD models--an alcoholic experience. *Int J Clin Pharmacol Ther.* 1997;35:465-468.
66 Zoethout RW, Schoemaker RC, Zuurman L, van PH, Dahan A, Cohen AF, et al. Central nervous system effects of alcohol at a pseudo-steady-state concentration using alcohol clamping in healthy volunteers. *Br J Clin Pharmacol.* 2009;68:524-534.
67 Martin CS, Moss HB. Measurement of acute tolerance to alcohol in human subjects. *Alcohol Clin Exp Res.* 1993;17:211-216.
68 Schweizer TA, Vogel-Sprott M. Alcohol-impaired speed and accuracy of cognitive functions: a review of acute tolerance and recovery of cognitive performance. *Exp Clin Psychopharmacol.* 2008;16:240-250.
69 Spear LP. Adolescent neurobehavioral characteristics, alcohol sensitivities, and intake: Setting the stage for alcohol use disorders? *Child Dev Perspect.* 2011;5:231-238.
70 Silveri MM, Spear LP. Decreased sensitivity to the hypnotic effects of ethanol early in ontogeny. *Alcohol Clin Exp Res.* 1998;22:670-676.
71 Ramirez RL, Spear LP. Ontogeny of ethanol-induced motor impairment following acute ethanol: assessment via the negative geotaxis reflex in adolescent and adult rats. *Pharmacol Biochem Behav.* 2010;95:242-248.
72 Spear LP. Assessment of adolescent neurotoxicity: rationale and methodological considerations. *Neurotoxicol Teratol.* 2007;29:1-9.
73 Acheson SK, Stein RM, Swartzwelder HS. Impairment of semantic and figural memory by acute ethanol: age-dependent effects. *Alcohol Clin Exp Res.* 1998;22:1437-1442.
74 Weafer J, Fillmore MT. Acute tolerance to alcohol impairment of behavioral and cognitive mechanisms related to driving: drinking and driving on the descending limb. *Psychopharmacology (Berl).* 2012;220:697-706.
75 Hiltunen AJ. Acute alcohol tolerance in cognitive and psychomotor performance: influence of the alcohol dose and prior alcohol experience. *Alcohol.* 1997;14:125-130.
76 Hiltunen AJ. Acute alcohol tolerance in social drinkers: changes in subjective effects dependent on the alcohol dose and prior alcohol experience. *Alcohol.* 1997;14:373-378.
77 Hurst PM, Bagley SK. Acute adaptation to the effects of alcohol. *Q J Stud Alcohol.* 1972;33:358-378.
78 Vogel-Sprott M, Barrett P. Age, drinking habits and the effects of alcohol. *J Stud Alcohol.* 1984;45:517-521.
79 Jones AW, Neri A. Age-related differences in the effects of ethanol on performance and behaviour in healthy men. *Alcohol Alcohol.* 1994;29:171-179.
80 Smith GT, McCarthy DM, Goldman MS. Self-reported drinking and alcohol-related problems among early adolescents: dimensionality and validity over 24 months. *J Stud Alcohol.* 1995;56:383-394.
81 Lintonen T, Ahlstrom S, Metso L. The reliability of self-reported drinking in adolescence. *Alcohol Alcohol.* 2004;39:362-368.
82 Maggs JL, Schulenberg JE. Initiation and course of alcohol consumption among adolescents and young adults. *Recent Dev Alcohol.* 2005;17:29-47.
83 Berten H, Cardoen D, Brondeel R, Vettenburg N. Alcohol and cannabis use among adolescents in Flemish secondary school in Brussels: effects of type of education. BMC *Public Health.* 2012;12:215.
84 Hibell B., Guttormsson U, Ahlstrom S, Balakireva O, Bjarnason T, Kokkevi A, et al. The 2007 ESPAD report: substance use among students in 35 countries. The Swedish Council for Information on Alcohol and Other Drug and the Pompidou Group of the Council of Europe,Stockholm . 2009.
85 Currie C, Gabhainn SN, Godeau E, Roberts C, Smith R, Currie D, et al. Inequalities in young people's health: Health Behaviour in School-aged Children (HBSC) international report from the 2005/206 survey. Health Policy for Children and Adolescents No. 5WHO Regional Office for Europe, Copenhagen, Denmark. 2008.
86 Weiss JC. The teen driver. *Pediatrics.* 2006;118:2570-2581.
87 Ramchandani VA, Plawecki M, Li TK, O'Connor S. Intravenous ethanol infusions can mimic the time course of breath alcohol concentrations following oral alcohol administration in healthy volunteers. *Alcohol Clin Exp Res.* 2009;33:938-944.
88 Plawecki MH, Zimmermann US, Vitvitskiy V, Doerschuk PC, Crabb D, O'Connor S. Alcohol exposure rate control through physiologically based pharmacokinetic modeling. *Alcohol Clin Exp Res.* 2012;36:1042-1049.

TABLE 1 Overview of adult studies of which data were incorporated in the PK/PD model development.

Ref	Study title	Ethanol treatment(s)	Placebo control for ethanol effect?	PD parameters in study which were also included in adolescent study
33	A Double-Blind, Randomized, Placebo- and Active-Comparator, Controlled, Triple-Dummy, 2-Period Crossover Study to Investigate the Psychomotor and Cognitive Effects of MK-0869 and Ethanol in Healthy Subjects	Constant ethanol infusion of 50 g/hr. Duration of infusion up to 1 hr (infusion terminated when blood alcohol concentrations exceeds 1.2 g/L)	No	N/A*
28	A placebo-controlled study on the effects of a novel method for alcohol infusion by clamping of breath alcohol concentration	Ethanol clamping; 5 hours at blood alcohol concentrations of 0.6 g/L	Yes	Adaptive tracking, Saccadic eye movement, Smooth pursuit
30	A double-blind, double-dummy, randomized, placebo-controlled, 3 period crossover study to investigate the effects of ethanol and L-000830982 on essential tremor	Ethanol clamping; 4 hours at blood alcohol concentrations of 0.6 g/L	Yes	--
29	Ethanol clamping at two levels of breath alcohol concentrations in Caucasian and Japanese subjects	Ethanol clamping; 5 hours at blood alcohol concentrations of 0.3 g/L and 0.6 g/L	Yes	Adaptive tracking, Saccadic eye movement, Smooth pursuit, VAS Bond and Lader, VAS alcohol effect, Body Sway
31	A randomized, double blind, placebo-controlled, double dummy, three-way crossover study to investigate the effects of both an intravenous ethanol clamp and a target controlled morphine infusion on resting state functional magnetic resonance imaging in healthy male volunteers	Ethanol clamping; 2.5 hours at blood alcohol concentrations of 0.6 g/L	Yes	VAS Bond and Lader, VAS alcohol effect
32	GSK598809 and Ethanol-Interaction study	Ethanol clamping; 5 hours at blood alcohol concentrations of 0.6 g/L	Yes	Adaptive tracking, Saccadic eye movement, Smooth Pursuit, VAS Bond and Lader, VAS alcohol effect, Body sway
34	ACT078573 and ethanol interaction	Ethanol clamping; 5 hours at blood alcohol concentrations of 0.6 g/L	Yes	Adaptive tracking, Saccadic eye movement, Smooth Pursuit, VAS Bond and Lader, VAS alcohol effect, Body sway
35	A double-blind, randomized, placebo-controlled, three-way crossover study to investigate the drug-drug interactions of brivaracetam and ethanol in healthy subjects	Ethanol clamping; 5 hours at blood alcohol concentrations of 0.6 g/L	No	Adaptive tracking, Saccadic eye movement, Smooth pursuit, VAS Bond and Lader, VAS alcohol effect, Body Sway, VVLT

Only tasks performed multiple times during a single occasion were included in the exploratory meta-analysis of PD parameters (i.e., VVLT data were not included). *Not applicable, as pre-dose alcohol concentrations (used to determine target level) were used for development of the PK model and therefore no pd was included in the PK/PD model.

TABLE 2 Summary of pharmacodynamic effects of alcohol compared to placebo (adolescent study)

	LS Means		Contrast
Parameter	Placebo	Alcohol	Alcohol vs Placebo Estimates of difference, 95% CI and *p*-value
Body sway (mm)	236.1	276.1	16.9% (5.2%, 30.0%) p=0.0069
Saccadic Inaccuracy (%)	6.22	6.27	0.05 (-0.65, 0.75) p=0.8728
Saccadic Peak Velocity (deg/sec)	454.1	452.5	-1.5 (-9.3, 6.2) p=0.6766
Saccadic Reaction Time (sec)	0.207	0.207	-.001 (-.006, 0.005) p=0.8502
Smooth Pursuit (%)	43.1	42.4	-0.7 (-2.3, 0.9) p=0.3744
Adaptive tracking (%)	26.36	25.48	-0.88 (-2.14, 0.39) p=0.1575
VAS Alertness (mm)	53.4	52.3	-1.1 (-2.4, 0.2) p=0.0879
VAS Calmness (mm)	55.5	55.4	-0.1 (-2.0, 1.8) p=0.9150
VAS Mood (mm)	56.6	57.1	0.5 (-0.5, 1.4) p=0.2946
VAS Alcohol effect (log(mm))	0.329	0.464	0.135 (0.033, 0.237) p=0.0134
Heart rate (beats per minute)	68.5	72.7	4.3 (2.6, 5.9) p<.0001
Diastolic blood pressure (mmHg)	61	61	-0(-3, 2) p=0.6988
Systolic blood pressure (mmHg)	116	113	-3 (-6, -0) p=0.0377
Correct acoustic stimuli at 650 msec	17.7	17.7	0.0 (-0.2, 0.2) p=0.8473
Average RT of visual stimuli (msec)	785.2	802.7	2.2 (-1.1%, 5.7%) p=0.1795
Correct visual stimuli at 650 msec	16.7	16.8	0.1 (-0.2, 0.4) p=0.6275
Correct visual stimuli at 150 msec	17.1	17.1	0.1 (-0.2, 0.3) p=0.5380
Correct visual stimuli at 50 msec	17.2	17.1	-0.1 (-0.4, 0.2) p=0.4435
Average RT of acoustic stimuli at 650 ms (msec)	638.64	644.21	0.9% (-7.3%, 9.8%) p=0.8296
Delayed word recall correct	15.9	15.1	-0.8 (-2.9, 1.2) p=0.3871
Word recognition correct	26.6	26.0	-0.6 (-2.1, 0.9) p=0.3917
Word recognition RT correct (msec)	857.1	837.3	-2.3% (-10.5%, 6.7%) p=0.5798
Word recall correct trial 1	11.0	9.4	-1.5 (-3.6, 0.6) p=0.1453
Word recall correct trial 2	15.9	14.3	-1.6 (-3.8, 0.6) p=0.1460
Word recall correct trial 3	18.8	18.0	-0.8 (-2.3, 0.6) p=0.2382

TABLE 3 Overview of study demographics among studies from which data were used of development of a PK/PD model

Ref	Number of subjects	Gender (male/ female)	Age (years)	Weight (kg)	Height	Study population
33	20	9/11	26.4 ± 9.2	75.1 ± 12.8	1.75 ± 0.08	Healthy adults
28	12	6/6	21.8 ± 6.1	75.5 ± 11.1	1.74 ± 0.10	Healthy adults
30	9	7/2	47 ± 21.3	81.0 ± 10.8	1.75 ± 0.11	Essential tremor patients
29	24	24/0	26.9 ± 5.6	76.1 ± 14.3	1.80 ± 0.09	12 Caucasian and 12 expatriate Japanese healthy adults
31	11	11/0	22.2 ± 2.2	80.8 ± 12.6	1.85 ± 0.08	Healthy adults
32	18	10/8	34.2 ± 14.4	72.4 ± 12.3	1.76 ± 0.09	Healthy adults
34	21	10/11	33.9 ± 15.6	73.9 ± 11.0	1.75 ± 0.08	Healthy adults
35	17	17/0	30.5 ± 8.3	81.7 ± 12.3	1.81 ± 0.07	Healthy adults
Adolescent study	17	8/9	17.1 ± 0.6	66.4 ± 8.6	1.76 ± 0.11	Healthy adolescents
Total	149	102/47	28.4 ± 12.6	75.4 ± 12.5	1.78 ± 0.09	

TABLE 4 Population parameter estimates of final PK model

Population parameters	Estimate [%RSE]	IIV (CV%)
vm (θ_1) (g/hr)	9.21 [3.6]	27.5
Km (θ_2) (g/L)	0.051 [26.2]	NE
v2 (θ_3) (L)	12.1 [3.0]	18.0
v3 (θ_4) (L)	33.8 [2.0]	29.3
Q (θ_5) (hr^{-1})	56.8 [3.0]	14.5
K_A(θ_6) (hr^{-1})	1.59 [7.6]	NE
Power of LBW on vm (θ_7)	0.692 [8.3]	
Power of LBW on v3 (θ_8)	0.407 [12.9]	
Power of age on Q (θ_9)	-0.283 [25.7]	
Power of height on Q (θ_{10})	2.29 [20.5]	
Residual error, proportional (%CV)	2.87 [38.9]	
Residual error, additive (g/L)	0.031 [11.6]	

Parameter uncertainties and interindividual variability (IIV) are provided when applicable. NE=not estimated.

TABLE 5 Parameter estimates of best PK/PD model for Smooth Pursuit Performance

Population parameters	Estimate [%RSE]	IIV (CV%)
BL (MPRD)	NE	10.0
KE (θ_1)	0.304 [7.9]	60.7
Residual error, additive (smooth pursuit performance [%])	5.40 [8.9]	

Parameter uncertainties and interindividual variability (IIV) are provided when applicable. NE=not estimated.

TABLE 6 Parameter estimates of VAS Alertness PK/PD model

Population parameters	Estimate [%RSE]	IIV (SD)
BL (mm) (θ_1)	53 [0.68]	
MPRD on BL (θ_2)	0.708 [10.0]	
KE (mm / (g/L)) (θ_3)	9.4 [18.0]	13.23
Residual error, additive (mm)	4.29 [42.1]	
Residual error, proportional (%CV)	6.67 [67.9]	

RSE, relative standard error; SD, standard error.

FIGURE 1 Individual change from baseline smooth pursuit performance over time of study CHDR0502[29]. Treatments: placebo (Placebo); ethanol clamping at 0.3 g/L (Clamp0.3); ethanol clamping at 0.6 g/L (Clamp0.6). The clamping was continued for 300 minutes. Similar effects of ethanol were seen in other studies. Open circles represent data; solid black lines represent a Loess curve through these points.

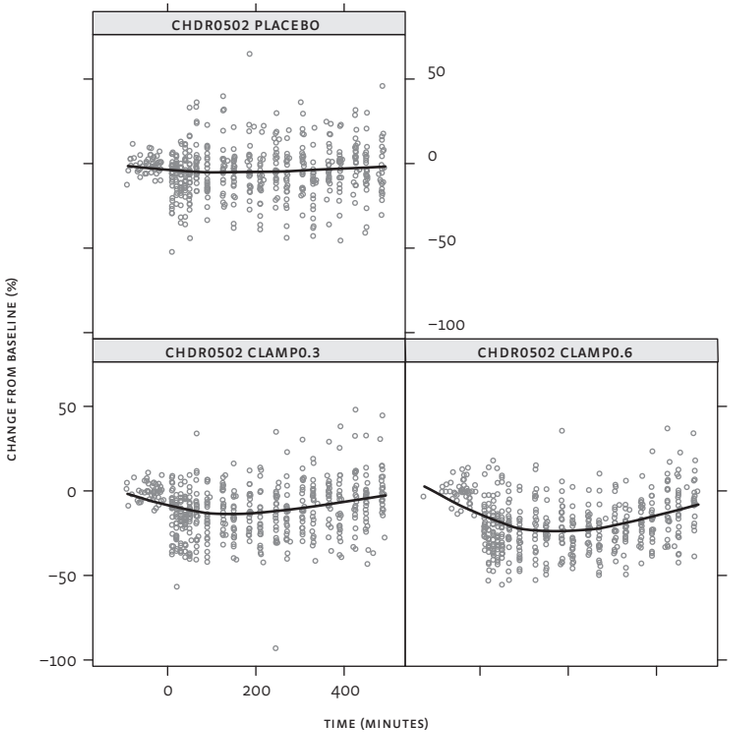


FIGURE 2 Ethanol concentration versus change from baseline smooth pursuit performance of CHDR0714[32], CHDR1011 (adolescent study), CHDR1214[35], CHDR0313[28], and CHDR0502[29]. 0.3 = target concentration of 0.3 g/L; 0.6 = target concentration of 0.6 g/L.

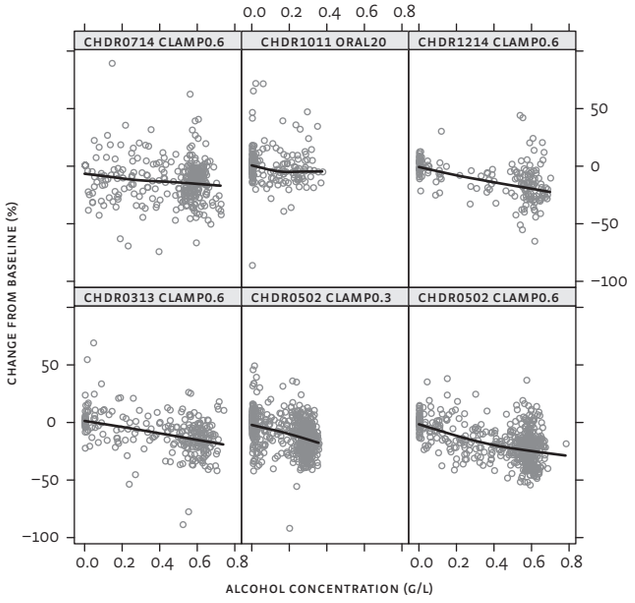


FIGURE 3 Schematic representation of the final PK model structure of ethanol

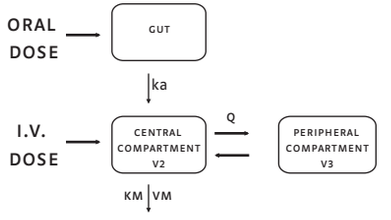


FIGURE 4 Individual predicted alcohol concentrations (IPRED) versus observations (DV). A linear regression of IPRED vs. DV is plotted as a continuous line.

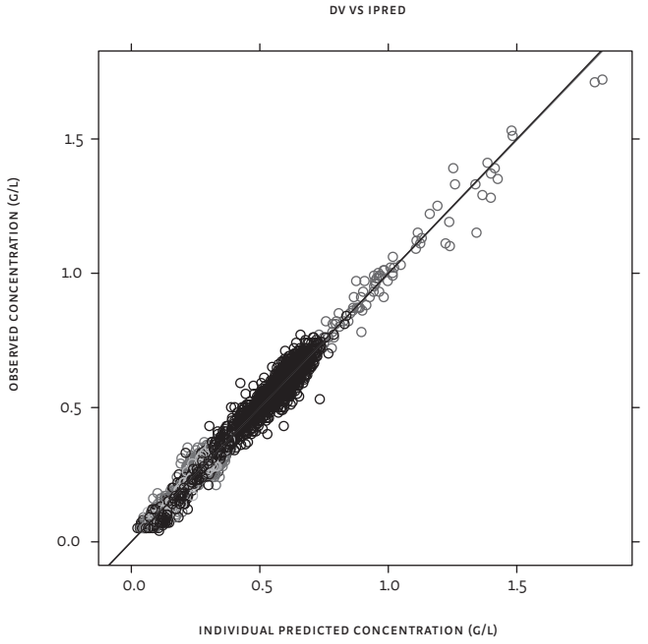


FIGURE 5 Goodness of fit plots of final PK model with Loess curve. Conditional Weighted Residuals (CWRESI) vs. individual predicted concentrations (IPRED) (upper panel); CWRESI vs. Time (lower panel).

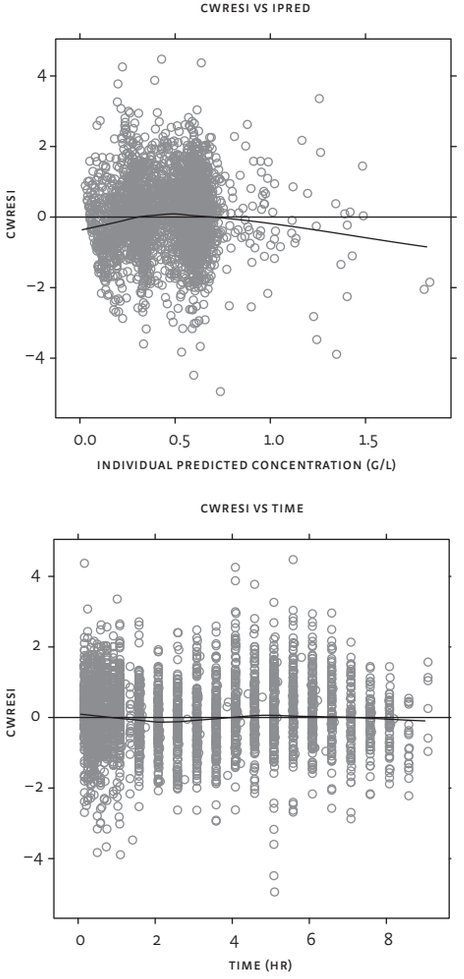


FIGURE 6 Distribution of ETAs. The continuous line represents a normal distribution with the same mean and standard deviation as the ETA. Corresponding parameters are: ETA 1: Q, ETA 2: Vm, ETA 3: V2 and ETA 4: V3.

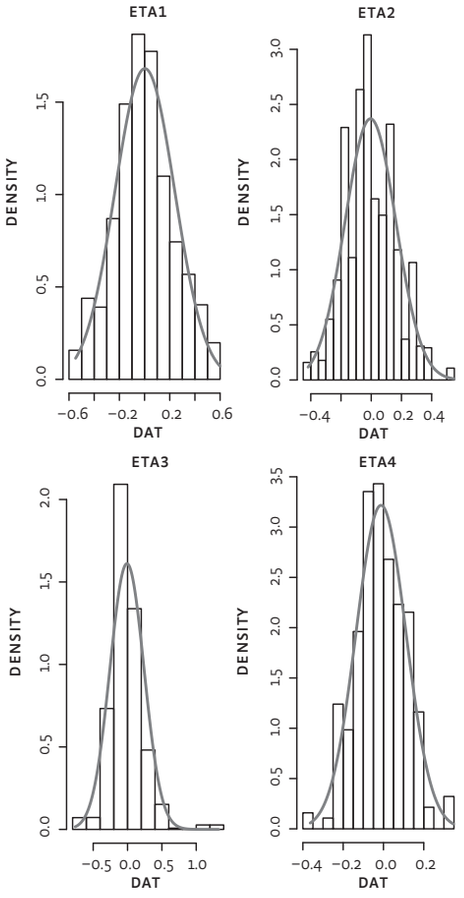


FIGURE 7 Individual predicted smooth pursuit performance (IPRED) versus observations (DV). A linear regression of IPRED vs. DV is plotted as a continuous line.

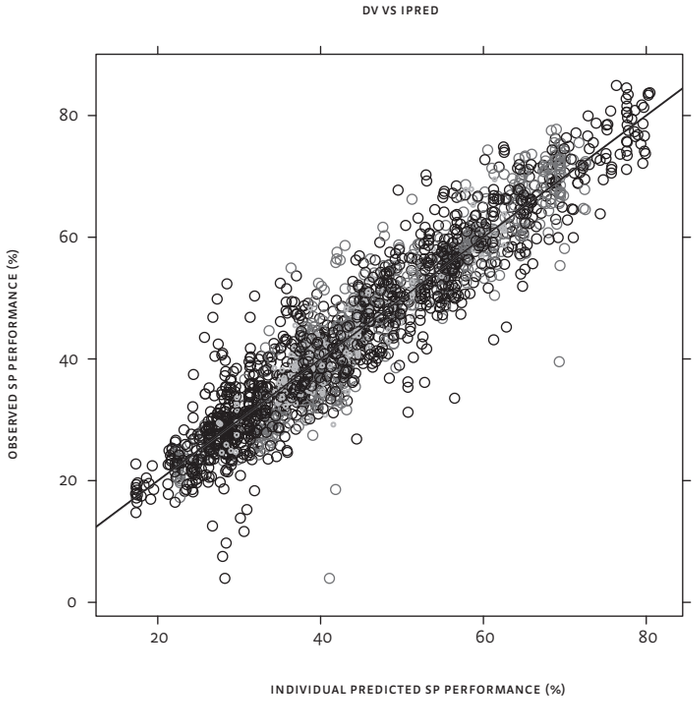


FIGURE 8 Goodness of fit plots of final PK/PD model for Smooth pursuit performance. Shown are conditional weighted residuals (CWRESI) versus IPRED (upper), time (middle) and ethanol concentration (lower panel).

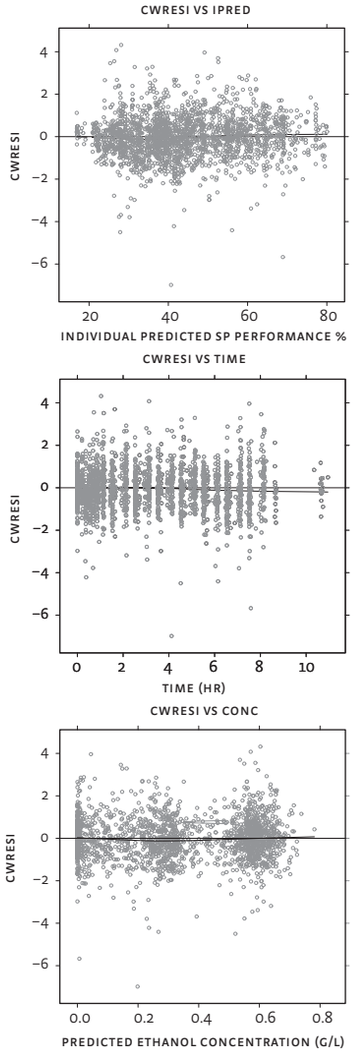


FIGURE 9 Distribution of ETAS. The continuous line represents a normal distribution with the same mean and standard deviation as the ETA. Corresponding parameters are: ETA 1: BL, ETA 2: KE.

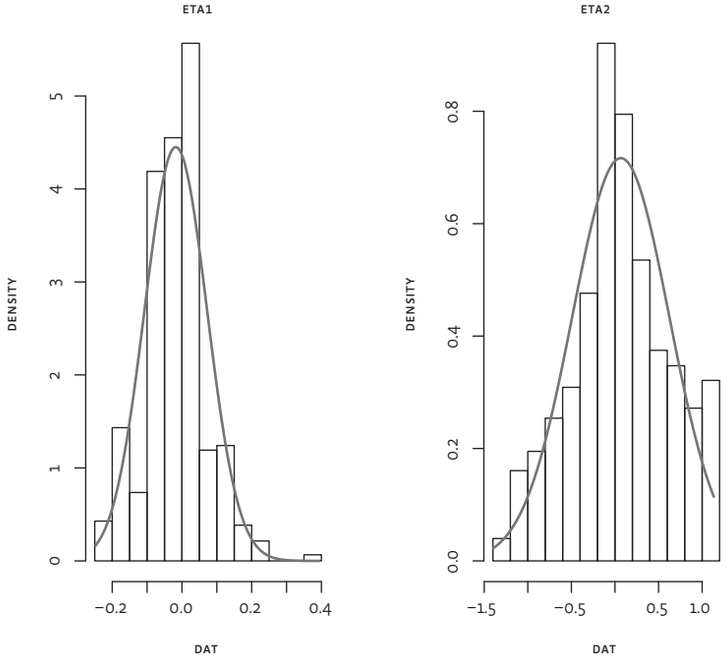


FIGURE 10 PD CWRESI over PK CWRESI plots, per study. PK data was matched to the PD by closest time point (open circles). A Loess curve was drawn through the data points (solid black line). Studies: 502=CHDR0502[29], 714= CHDR 0714[32], 1011= CHDR 1011 (adolescent study), 1214= CHDR 1214[35], 313= CHDR 0313[28].

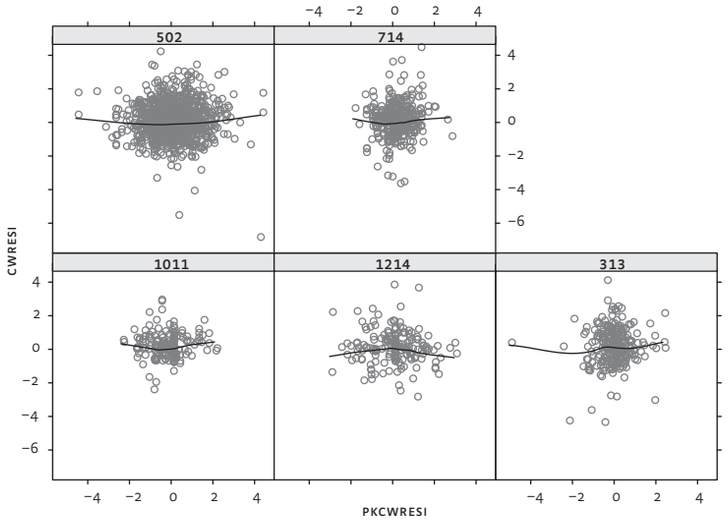


FIGURE 11 Individual predicted VAS Alertness score (IPRED) versus observations (DV). A linear regression of IPRED vs. DV is plotted as a solid black line.

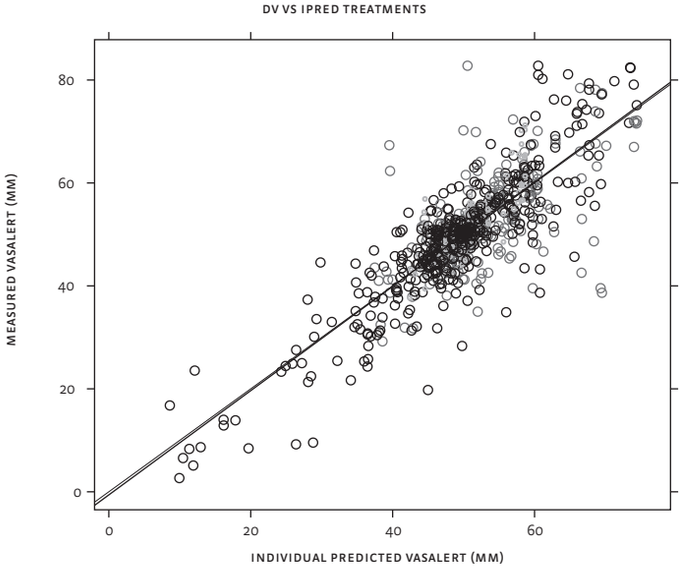


FIGURE 12 Goodness of fit plots of VAS Alertness PK/PD model. Shown are conditional weighted residuals (CWRESI) versus IPRED (upper), time (middle) and ethanol concentration (lower). A Loess curve (solid black line) was drawn through the data points (open circles).

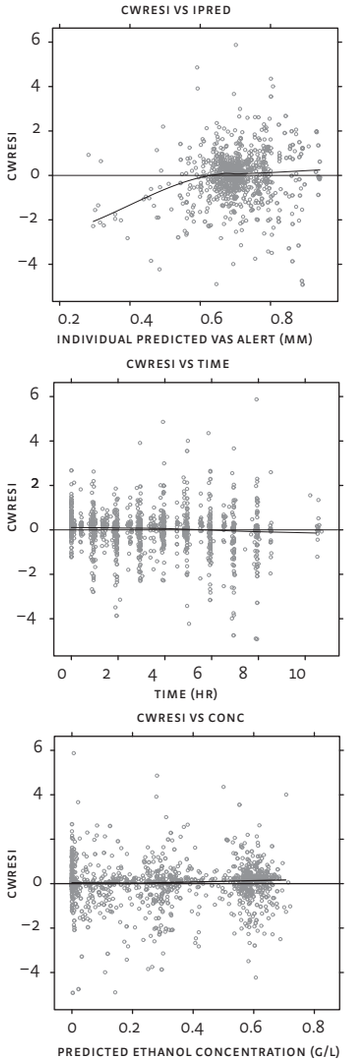
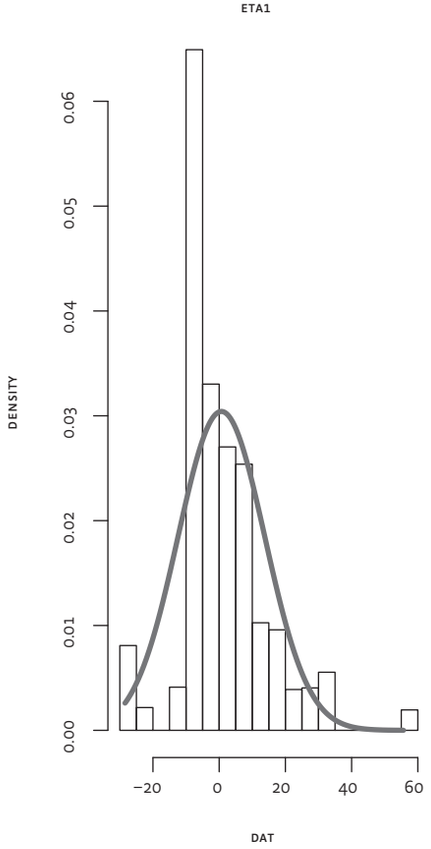


FIGURE 13 ETA distribution. The black line shows a normal distribution with the same mean and standard deviation as the ETA. Corresponding parameter of ETA 1 is KE.



SUPPLEMENTARY DATA

FIGURE 1 **Smooth pursuit LSMs change from baseline profile with 95% CI as error bars.**

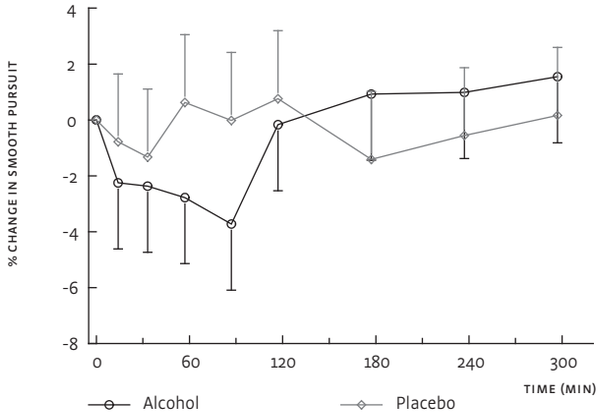


FIGURE 2 **VAS alertness LSMs change from baseline profile with 95% CI as error bars.**

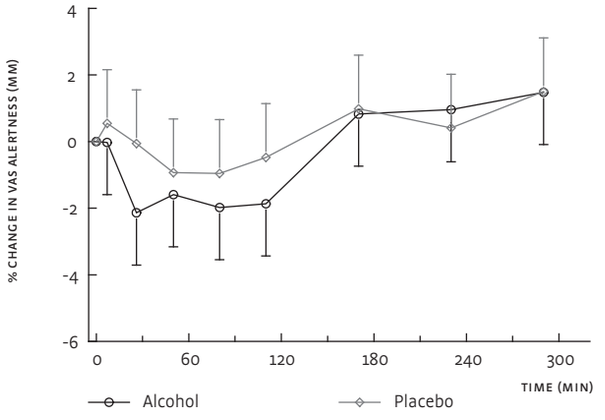


FIGURE 3 **VAS alcohol effect LSMs change from baseline with 95% CI error bars.**

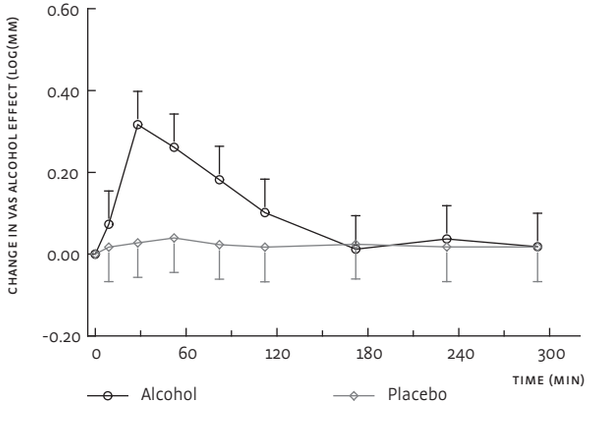


FIGURE 4 **Body sway LSMs change from baseline with 95% CI error bars.**

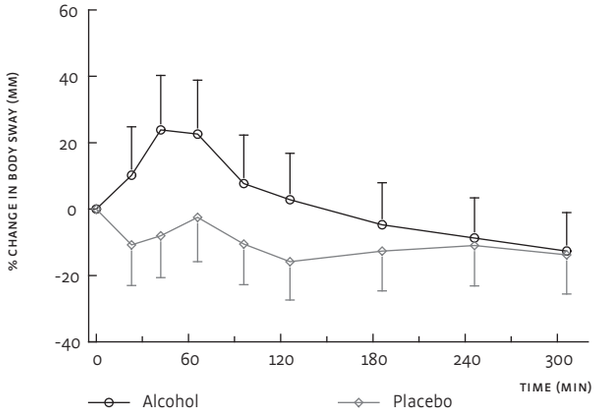


FIGURE 5 **Heart rate LSMs change from baseline with 95% CI error bars.**

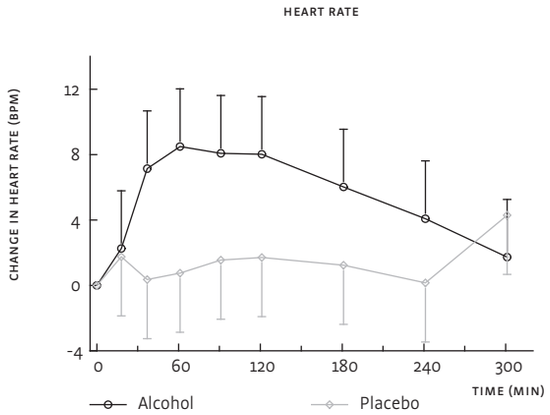
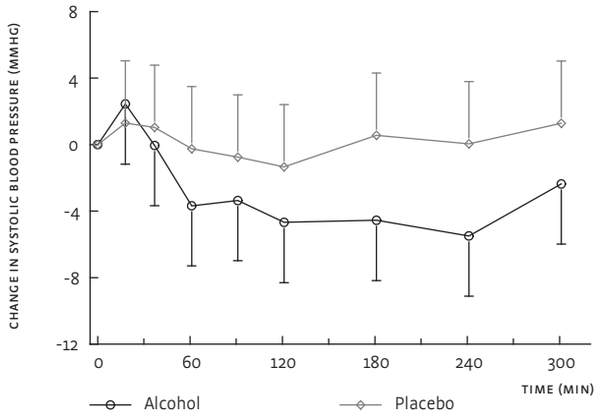


FIGURE 6 **Systolic blood pressure LSMs change from baseline with 95% CI error bars.**



CHAPTER 8

Pharmacokinetics of prolonged-release melatonin mini-tablets in children with both autism spectrum disorder and a sleep disorder

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ABSTRACT

Sleep problems are highly prevalent among children with autism spectrum disorder (ASD), and these problems can significantly impact both the child and the child's family. Because conventional therapies are generally unsatisfactory, there is currently an urgent need for an effective intervention. Melatonin is effective for ameliorating sleep problems in this pediatric population, and its favorable short-term and long-term risk profile has led to the increasingly widespread clinical use of melatonin as an off-label medicine. Currently, no age-appropriate prolonged-release melatonin preparation is commercially available. Here, we performed a pharmacokinetics crossover study of Circadin 1 mg mini-tablets, a prolonged-release melatonin formulation, in 16 children and adolescents with both ASD and a sleep disorder. We tested 2 mg and 10-mg doses of Circadin based on the dose range we will use in an upcoming efficacy trial. Whole saliva samples were collected using passive drool collection, and melatonin concentration was measured in these samples. Adverse events were monitored throughout the study, and potential sedative effects were assessed using the Observer's Assessment of Alertness/Sedation (OAA/S) scale for 10 hours after administration. Pharmacokinetics parameters for melatonin were estimated using non-compartmental modeling. All 16 subjects (age range: 7-15 years) had a clinical diagnosis of autism spectrum disorder (based on DSM-IV-TR criteria). Mini-tablets were found to be both safe (i.e., none of the children choked) and acceptable to the children. The melatonin concentration peaked within two hours of administration and remained elevated for several hours thereafter. Circadin exposure was dose-linear, and clearance (1,000 L/hr) was similar between the dose groups. The median apparent terminal half-life was comparable between dosages. All reported side effects were consistent with known side effects. The highest levels of sedation (assessed using the OAA/S) were observed between 2 and 3 hours after administration of Circadin 2 mg and between 2 and 6 hours after administration of Circadin 10 mg. In conclusion, this study demonstrates the short-term safety, acceptability, and prolonged-release profile of Circadin mini-tablets in 16 school-age children and adolescents with ASD.

Introduction

Sleep problems are highly prevalent among children with autism spectrum disorder (ASD)[1,2], a very common spectrum of neurodevelopmental disorders that is characterized by impaired communication and social interaction together with restrictive and repetitive behaviors. Increased sleep latency, waking during the night, and difficulty awakening in the morning are among the most frequently reported problems in children with ASD[3–6]. These sleep problems can become prominent as early as two years of age, and they can persist for many years. Sleep disorders in these children can place a significant burden on the child's physical and mental health, thereby negatively affecting performance in school and creating stress for the child's family[7–12]. In addition, these problems are usually resistant to conventional sleep medications (e.g., benzodiazepines) or antipsychotic agents[12,13] – which in itself can cause adverse side effects or can lead to interactions with other drugs, and their use is contraindicated in some cases. Combining these drugs with behavioral approaches – which are generally difficult to apply, time-consuming, and usually require skilled expertise – is often unsatisfactory. Thus, there is an urgent need for an effective intervention[3,14].

Chronic sleep-wake disorders in children with ASD are associated with an inability to synchronize the circadian rhythm-generating biological system with environmental *zeitgebers*, thus resulting in abnormal melatonin secretion[15,16]. Moreover, a shift in peak melatonin secretion may underlie sleep-onset problems, whereas reduced rhythm amplitude may underlie sleep interruptions and early morning awakening[4]. Melatonin's efficacy in ASD was recently classified as grade A[17]. These findings – together with the observation that melatonin has a highly favorable short-term and long-term risk profile – have led to the increasingly widespread clinical use of melatonin as an off-label medicine. However, the quality of many melatonin preparations is questionable, as these preparations are available as a health supplement in some countries (for example, in the United States) and are therefore not necessarily manufactured in accordance with good manufacturing practice. In addition, no prolonged-release preparation for use in children is commercially available, even though prolonged-release preparations are preferred over fast-release preparations; prolonged-released formulations provide sustained blood levels[18] and are more useful for maintaining sleep[19]. Importantly, although young children may be able to swallow small tablets, they cannot swallow large tablets, and children with ASD can have difficulty swallowing tablets at any age. Therefore, there is a need for a reliable, commercially available, age-appropriate prolonged-release melatonin preparation that is licensed for use in children with ASD and sleep problems.

Circadin 2 mg is a prolonged-release melatonin formulation that is licensed in the EU for treating primary insomnia in patients 55 years of age and older. Previous studies demonstrated that Circadin 2 mg is both safe and efficacious in children and adolescents with neurodevelopmental and behavioral disorders[19–21]. In order to provide its intended release profile, Circadin tablets must be swallowed whole. An age-appropriate formulation was developed in the form of mini-tablets, which have the same *in vitro* dissolution profile as regular-size Circadin tablets and should therefore produce a concentration-time profile that mimics the physiological rhythm (depending on the amount and timing of the dose).

Here, we performed a pharmacokinetics (PK) crossover study in children with both ASD and a sleep disorder. This initial study will be followed by a randomized, double-blind, placebo-controlled study to investigate the efficacy and long-term safety of Circadin mini-tablets in patients with a neurodevelopmental disorder. To minimize study-related burden and risk, melatonin concentration was measured non-invasively from saliva samples. Prior to the study, PK modeling and simulations were performed in order to determine the minimum number of sampling time points that would be needed and to adapt the sampling time points to accommodate the subjects as much as possible.

Methods

Subjects

Sixteen children with a DSM-IV-TR diagnosis of autistic spectrum disorder (ASD) with current sleep disorder were recruited from specialized child mental health clinics in the Netherlands. Current sleep disorder was defined as difficulty initiating or maintaining sleep, or non restorative sleep, for at least 1 month (DSM-IV 307.42). Subjects with an untreated medical or psychological condition that could be the etiology of sleep disturbances (e.g., restless leg syndrome) were not allowed to participate. The subject had to be able to comply with taking the study drug, collaborate freely with the study procedures and understand instructions in Dutch. Written informed consent was obtained prior to study initiation from parents having parental responsibility or from the legal guardian(s). In children aged 12 years or older, the written informed consent of the child was needed in addition to that of parents having responsibility/legal guardian. We excluded subjects with a history of difficulty with swallowing or easy choking, current symptoms suggestive of obstructive sleep apnea syndrome or any breathing related sleep disorders or periodic limb movements, or non-stable epileptic attacks within 3 months prior to screening (in case of a history of epilepsy). Other exclusion criteria included known clinically significant disturbance(s) in hepatic and/or renal function, current asthmatic symptoms, clinically relevant periodontal disease and/or oral injuries, pregnancy (at time of screening), known allergy to melatonin, and unstable use of allowed medication within 2 months prior to the screening. The subjects were not allowed to use any form of melatonin within one week prior to dosing. In addition, other concomitant medication was also not allowed within one week prior to study days, except for paracetamol, oral contraceptives or some topical medication (i.e., creams, ointments, gels or lotions used to induce a local effect without systemic exposure). Drugs or dietary supplements that can cause pharmacokinetic interactions with melatonin (by inhibition or induction of involved CYP450 isoenzymes), can alter melatonin secretion or release (like (in)direct beta-sympathicomimetic or -sympathicolytic drugs) or affect melatonin's mode of action (like $GABA_A$ receptor modulators and antidepressants) were not allowed from one week to the first occasion, throughout the study. The subjects were allowed to continue the use of the antipsychotics aripiprazole and risperidone, and of non-enzyme-inducing antiepileptic drugs. Stimulant drugs (methylphenidate, dexamphetamine) had to be discontinued at least 5 times the half-life of the specific drug to ensure complete wash-out during study days. On study days, the subjects had to refrain from aspirin use or drugs that contain ibuprofen. The subjects had to refrain from consumption of caffeine-containing products for 24 hours before each occasion until the end of the study day. On study days, the subjects had to refrain from eating bananas and chocolate and from drinks containing articifical colorants, caffeine or alcohol during the entire day. In addition, administration of medication and caffeine was questioned and a urine drug screen and pregnancy test were performed before any study-related procedures were started. The Central Committee on Research involving Human Subjects approved the study protocol.

Study design

This was an open label, single ascending dose, crossover study of 2 and 10 mg Circadin mini-tablets. The first occasion included a 24-hour baseline measurement day. This study followed a staggered approach: the study was completed in at least four children aged 11-17 years before commencing in the group of children 10 years of age or younger. Pubertal stage was assessed at screening. Female subjects were not studied during the stop week of their oral contraceptives. The subjects were confined to the clinical research unit for approximately 24 hours after melatonin administration. Subjects were given a 'paskaart' (stopping card) which he or she could show to indicate a study related activity should be stopped. The use of this card was explained during the screening and start of each occasion. The 'Gedragscode verzet minderjarigen' (Code of conduct in case of resistance in minors) and 'Gedragscode verzet

bij mensen met een verstandelijke handicap' (Code of conduct for physicians involved in the assessment of expressions of objection by people with mental disabilities) were to be followed in case a subject displayed resistance against any study related activity.
As this was an exploratory pharmacokinetic study in children and adolescents with ASD, no formal power calculation was performed. However, based on previous experience, it was expected that a sample size of 16 subjects would suffice for determination of PK parameters in saliva.

Interventions

Circadin mini-tablets contain 1 mg of the active substance N-acetyl-5 methoxy-tryptamine, which is identical to endogenous melatonin. The mini-tablets were produced via traditional tableting methods, including dry-mixing and direct compression. A coating was applied to enhance palatability and reduce susceptibility to light (as melatonin is light sensitive). Most of the published studies as well as off-label use in clinical practice have shown that in the pediatric population melatonin efficacy is achieved in a certain range of doses, mainly 2-12 mg. Therefore, a unit dose of 1 mg was chosen to allow maximal flexibility in dose modification. Based on the anticipated dose range in the subsequent efficacy trial, dosages of 2 and 10 mg were included. In clinical practice, Circadin is taken in the evening, 1-2 hours before the child's usual bedtime. However, in order to avoid sleep disturbance due to repeated nighttime saliva sampling after melatonin administration, it was decided to administer melatonin during daytime (in the morning) instead of in the evening to minimize burden related to study participation. PK simulation indicated that the PK profile could be accurately evaluated after daytime administration (see Figure 1).

Circadin was administered in the morning immediately after a standardized breakfast. Administration was done in sitting position and in bright light (≥2,500 lux). To ensure that the mini-tablets were ingested in whole, mini-tablets could be taken together with standardized amounts of strawberry jam (5 ml), strawberry yoghurt (5 ml), orange juice (10 ml), semi-skimmed milk (10 ml) or water (10 ml). Melatonin has been shown to be stable in these common liquids and foods tested for up to 6 hours at room temperature (no degradation peak)[22]; hence it was unlikely that mixing the mini-tablets with these vehicles would affect melatonin dose. When mini-tablets were mixed in such a vehicle, it was recommended that it should be administered to the subject immediately. If immediate administration was not possible, the mixture was thrown away and a fresh dose was prepared using reserve mini-tablets. If the mini-tablets were administered mixed with a vehicle on the first occasion, mini-tablets were also administered in the same vehicle on the second occasion.

Swallowing was carefully observed. After deglutition the subject's mouth was inspected by the investigator using a flashlight and outcome of drug administration was scored as following[23]: (1) swallowed (no chewing during deglutition and no solid residuals found during oral inspection); (2) chewed (swallowed most of the tablet pieces, but small residuals found during oral inspection); (3) spat out (no observed deglutition and no solid found during oral inspection); (4) choked on (the mini-tablet was inhaled or a cough was caused during swallowing), or (5) refused to take (all actions preventing the physician/(co-)investigator/caretaker placing the mini-tablet in the mouth). In case of unsuccessful administration (e.g., spitting or visible chewing without other clear signs of obstruction), an attempt was made to administer a new dose using reserve mini-tablets available for the occasion. In case of visible chewing, the occasion was terminated and the new dose was given at least 7 days later. In addition, a saliva sample was obtained two minutes after ingestion, for determination of elevated melatonin concentrations (compared to pre-dose levels) due to contamination.
Standardized meals were provided following drug administration. Water (200 ml) was given every 2 hours post-dose to maintain all subjects on a uniform hydration schedule.

The wash-out period in-between occasions was at least 7 days and melatonin was administered at the same time of day in all subjects to avoid confounds from circadian variability.

Pharmacokinetic methods

SALIVARY MELATONIN COLLECTION AND BIOANALYSIS

For children who normally fall asleep before 12 hours prior to melatonin administration, saliva samples for baseline melatonin concentration determination were collected on the first occasion at t= -24, -20, -16 hrs, just before normal time of falling asleep, at a time point after falling asleep but before t=-5 hrs when the child wakes up on its own, -5 hrs, immediately after waking up, and 0 hrs pre-dose (not more than 10 min before dosing). For children who normally fall asleep after 12 hours prior to melatonin administration, baseline samples were collected at t= -24, -20, -16, -12 hrs, just before normal time of falling asleep, -5 hrs, immediately after waking up and 0 hrs pre-dose (not more than 10 min before dosing). Time points for PK sampling after administration were determined based on PK simulations (Figure 1). Whole saliva samples were collected by passive drool using the Saliva Collection Aid (Salimetrics Europe), a collection aid designed to simplify collection of whole saliva, hereby increasing subject compliance. The vented design helps avoid sample foaming and the device is constructed of polypropylene to avoid sample retention or contamination. Absorption loss and analyte degradation were further minimized by the use of low-protein binding storage cryovials (Salimetrics, UK), which are suitable for use with this collection aid. A minimum sampling volume of 1 ml was needed. Subjects and caregivers were trained prior to the first occasion on how to collect saliva using this collection aid. As bright light can suppress endogenous melatonin production, collection of saliva samples was done in dim light (< 8 lux). Plasma and salivary melatonin concentrations have been reported to increase when moving from a supine to a standing position and decrease when these positions are reversed, due to changes in plasma volume[24]. Therefore, saliva samples were collected after spending at least 15 minutes in a sitting position. Deviations were allowed during the night in order to minimize the duration that subjects need to be awake for saliva sampling; in these cases, duration of time spent in sitting position was recorded as a note.

Subjects had to rinse their mouths out with water 10 minutes before each saliva collection and were instructed not to express mucus or sputum from the back of the throat into the collection tube. Saliva was collected in tubes wrapped in aluminum foil to protect the samples from light. After saliva collection, saliva was transferred to duplicate transport tubes (also wrapped in aluminum foil; approximately 1 ml per tube) and stored within 2 hours after collection at a maximum temperature of -20°C. Saliva melatonin concentrations were determined by ABL (Analytisch Biochemisch Laboratorium BV, Assen, The Netherlands) using a LC-MS/MS method (precision and accuracy, CV ≤ 15%; validated range, 2 – 20,000 pg/ml).

URINARY 6-SULPHATOXYMELATONIN CONCENTRATIONS

Urine samples were collected for determination of 6-sulphatoxymelatonin (6-SMT). Urine was collected after spontaneous voiding in potty-trained children or using a urine collection bag in prepubertal non-potty-trained children. All urine passed over 12 hour periods was collected into a standard urine bottle. The total volume was measured and recorded and approximately 5 ml was kept at -20°C. No preservative was required, as 6-SMT is stable in urine for one day at room temperature, 2 days at 4°C and for at least 2 years at -20°C. 6-SMT concentrations were determined by ABL (Analytisch Biochemisch Laboratorium BV, Assen, The Netherlands) using a qualified radioimmunoassay (^{125}I label) (Stockgrand Ltd.; validated upper limit of quantification, ULOQ 2,500 pg/ml).

Safety

At screening, pregnancy testing was done in females who had a menstrual cycle using a qualitative, color immuno-chromatografic detection of HCG in urine using CARDS O.S. H.C.G.-urine test kits (Pacific Biotech, Inc., San Diego, CA 92121, USA). Adverse events were monitored throughout the study.

Assessment of sedation

As Circadin was given in the morning, it was anticipated that daytime sleepiness and slight lowering of body temperature could occur. As children with ASD may be unable to verbally describe level of sedation, the Observer's Assessment of Alertness/Sedation (OAA/S) Scale was used pre-dose and 1, 2, 3, 6, and 10 hours after melatonin administration. This scale was developed to more objectively measure the level of alertness in subjects who are sedated and has been shown to be reliable and valid and to be sensitive to the effects of for example midazolam[25] and is comparable to the Ramsey scale which is commonly used in the pediatric intensive care unit to assess level of consciousness[26].

Statistical analysis

PHARMACOKINETIC ANALYSIS

Exploratory individual and summary concentration-time profiles of melatonin and 6-SMT were generated to identify potential outliers. Observations below the lower limit of quantification were excluded from the analysis. Saliva samples taken 2 minutes after dosing ('contamination samples') were excluded from analysis, as these were unlikely to reflect actual (excreted) melatonin saliva concentrations. PK parameters were estimated using R (v2.12.0, R Foundation for Statistical Computing, Vienna, Austria, 2010).

Results

Subjects

A total of 16 subjects (12 male, 4 female) with ASD were enrolled in 2013-2014. The average age was 10 years (range: 7-15 years), and the average weight was 40.6 kg (range: 26-67 kg). Most subjects were prepubescent (Tanner Stage 1, 50%), with fewer subjects having an intermediate pubescent stage (Stage 2, 19%; Stage 3 and 4, 6% each) or having adult features (Stage 5, 19%). Comorbidities included 22q11 deletion syndrome, migraine, asthma, eczema, ADHD, cystitis, and constipation. The medications that were continued during the study included propranolol (10 mg twice daily, by one subject), flixotide (250 µg twice daily, by two subjects), ventolin (200 µg as needed, by one subject), desloratine (1 mg twice daily, by one subject), macrogol (6.9 g once daily, by one subject), and an antibiotic treatment (unspecified, for cystitis, by one subject). Antipsychotic agents (aripiprazole, 1-3 mg once daily, by two subjects; and risperidone, 0.5 mg once daily, by three subjects) were also continued during the study. Any disallowed concomitant medication was discontinued prior to the study in accordance with the protocol. Seven subjects had used melatonin prior to the study. During the study, all administrations were successful, and none of the subjects choked or visibly chewed on the study medication. All of the subjects completed the study. Subject 9 (a 45-kg 13-year-old boy with Tanner Stage 5) and subject 10 (a 58-kg 15-year-old boy with Tanner Stage 5) received an incorrect melatonin formulation that did not comply with the intended *in vitro* release profile. Thus, the data obtained from these two subjects were excluded from the PK analysis and evaluation of the OAA/S scores; however, these two subjects were included in the safety analysis. Both of these subjects used melatonin prior to the study. In addition, it is suspected that three subjects (subjects 5, 6, an 11) held the tablets in their mouth for a long time after administration of 2 mg Circadin, resulting in measured melatonin levels at 1 hour which exceeded the levels of other subjects (up to 10-fold) probably because melatonin was released in their mouth before swallowing. These children were excluded from the sub-analysis PK population.

Concentration-time profiles of melatonin in saliva

For the endogenous melatonin concentrations measured prior to administration of the Circadin mini-tablets, each individual had several observations that exceeded the assay's lower limit of quantification (LLOQ; i.e., >2 pg/ml). For 14 out of 16 subjects, no saliva samples were taken from 23:00 (11:00 PM) through 7:00 AM in order to minimize patient burden.

In one subject (subject 4), the melatonin concentration measured in the saliva sample taken at t=3 hours exceeded the assay's upper limit of quantification (ULOQ; i.e., >20,000 pg/ml). Because the number of freeze/thaw cycles for this sample exceeded the number of validated cycles, this observation was excluded from further analysis. None of the other PK samples had a concentration that exceeded ULOQ. Three and 8 samples that were taken two minutes after administration of 2 and 10 mg ('contamination samples') had a concentration that exceeded the ULOQ.

The apparent terminal elimination rate constant and its derived parameters – including the apparent terminal half-life, the area-under-the-curve (AUC) from dosing to infinity (including extrapolated percentages), the apparent drug clearance rate, and the apparent volume of distribution – were excluded for subject 6 (on the 2 mg dosing occasion) and subject 14 (on both occasions), as this parameter could not be accurately estimated for these occasions based on the regression plots.

The individual concentration-time profiles are presented in Figure 2. The melatonin concentration peaked within two hours of administration and remained elevated for several hours thereafter (Figure 3). Circadin exposure (derived from the AUC data) was dose-linear, and apparent clearance (approximately 1,000 L/hr) was similar between the dose groups (Table 1). The median apparent terminal half-life was similar between the 2-mg and 10-mg dose groups. The apparent volume of distribution in the saliva decreased (with corresponding clearance) with increasing dose.

Urine 6-sulphatoxymelatonin concentrations

Urine 6-sulphatoxymelatonin concentrations in samples collected during the first 12 hours of administration exceeded ULOQ (i.e., >2,500 pg/ml) for 1/14 subjects following a 2-mg dose and for all 14 subjects following a 10-mg dose. The mean total 6-SMT recovered from urine during the baseline period was 4.2 µg/12 daytime hours and 13.5 µg/12 nighttime hours. Following administration with Circadin 2 mg, the mean amount of total 6-SMT recovered from urine was 989.5 µg/12 daytime hours and 95.3 µg/12 nighttime hours. Following administration with Circadin 10 mg, many of the 6-SMT values were above the ULOQ during the collection period 0-12 hours following dosing (further dilution of the samples was not validated).

Sedation level

Shortly after waking up in the morning (at the time point of the first assessment of the level of sedation), several subjects assessed by the Observer's Assessment of Alertness/Sedation Scale transitory scored 'drowsy/normal speech'; one subject was scored 'drowsy' just prior to the administration of Circadin 10 mg (Figure 4). Following morning administration of Circadin (2 mg or 10 mg), most subjects assessed by the Observer's Assessment of Alertness/Sedation Scale scored to have a mild increase in sedation. The highest levels of sedation were observed between 2 and 3 hours after administration of Circadin 2 mg and between 2 and 6 hours after administration of Circadin 10 mg, with up to 6/14 (2 mg) and 7/14 (10 mg) of children scoring 'drowsy to normal speech' or a higher sedation score.

Adverse effects

In the 16 subjects, the following adverse events were potentially or likely associated with the administration of 2 and/or 10 mg Circadin: fatigue (in seven and eight subjects after 2 and 10-mg doses, respectively), a sensation of heaviness (in one subject after a 2-mg dose), somnolence (in three and two subjects after 2 and 10-mg doses, respectively), falling asleep (in two subjects after both the 2 mg and 10-mg dose), headache (in one and three subjects after 2 and 10-mg doses, respectively), and nausea (in one subject after a 10-mg dose). All of the adverse events were transient and mild, and no serious adverse events were reported. Nausea, fatigue and headache were more frequently reported following Circadin 10 mg compared with Circadin 2 mg.

Questionnaire regarding participation

Overall, the subjects and their caregivers were positive about the burden and duration of the study. Nearly all subjects (69%) and caregivers (88%) stated that they would consider (consent for) participating in a similar study again. Most of the children did not find the saliva sampling to be bothersome. The four subjects who indicated that saliva sampling was somewhat bothersome were 10, 11, 13, and 15 years old. The one child who would not participate again found participating in the study rather nice, but found the study day duration long and the urine sampling a bit bothersome. The one parent who would not consent for participation in similar research found the child's participation very nice, but thought saliva and urine sampling were a bit bothersome, as did the child. The child itself found participating not so nice and did not know if it would participate again.

Discussion

This is the first study to investigate the pharmacokinetics (PK), acceptability, and short-term safety of a new prolonged-release melatonin formulation in children with ASD. The ability to deliver pediatric medicines accurately and safely is essential in order to ensure that the correct dose is received and that the formulation is safe, easy to use, and acceptable from both the child's and the caregiver's perspective. Due to age-related differences in the anatomy of the buccal cavity[27], young children may not be able to swallow a large tablet, and children with ASD or other neurodevelopmental disorder might experience difficulty swallowing a tablet at any age. Therefore, Circadin mini-tablets were developed as an age-appropriate formulation for this patient population. These 3-mm diameter mini-tablets were produced using traditional tableting methods (i.e., dry-mixing and direct compression) that can be reproduced easily and do not require sophisticated manufacturing equipment. A coating was applied to the tablets to enhance their palatability and to reduce the active ingredient's exposure to light. Previous studies showed that this type of formulation is safe for use in healthy pre-school and school-age children up to six years of age[28 23]. In our study, this formulation was ingested safely (i.e., none of the children choked) and was acceptable to the children. However, children who had difficulty swallowing or had a history of choking easily were excluded from the study; therefore, the results may not be directly applicable to this population.

Because Circadin mini-tablets have the same *in vitro* dissolution profile as regular-size Circadin tablets, the melatonin concentration-time profile of the mini-tablets was expected to mirror the profile of regular-size Circadin tablets. The PK properties of Circadin tablets have been investigated in both adults and children, as well as in children and adolescents with neurodevelopmental and behavioral disorders[19–21]. For our study, we chose 2-mg and 10-mg doses based on the anticipated dose range in an upcoming efficacy trial in which the children will initially receive 2 mg Circadin, after which they will have the option to modify the dose, first increasing to 5 mg, and then increasing to 10 mg if the lower doses are not efficacious. Based on the results of a recent randomized clinical trial in which the long-term effects and safety of melatonin were investigated in children[29], we anticipate that some of the children in our upcoming study will need a dose of 10 mg. Other studies[18,30] suggest that per kg body weight, children require a higher amount of melatonin than adults[19]; this difference may be due to the higher endogenous melatonin levels in healthy children, as prepubescent children metabolize melatonin faster than adults[31 32]. Ideally, the *in vivo* release of Circadin mini-tablets should peak within 2-4 hours of administration, and the levels should remain elevated for 4-5 hours thereafter. In our study, salivary drug concentrations were measured starting one hour after administration (and thereafter), and the oral cavity was rinsed after the mini-tablets were swallowed in order to minimize residual oral contamination. The melatonin concentration peaked within two hours following treatment with both 2 and 10 mg Circadin mini-tablets, and the concentration remained elevated for several hours thereafter. Circadin exposure in the saliva was dose-linear, and clearance in the saliva was similar

between the two dose groups. Determination of the melatonin metabolite, 6-SMT, in urine indicated extensive metabolism and excretion in the first 12 hours following dose administration. Consistent with previous research[33 34], melatonin PK in saliva varied widely. Given the fairly consistent shape of the individual saliva curves, and given the known variability of melatonin in plasma[33 34], this variability is likely due to variability in absorption and clearance, rather than variability in the saliva-to-plasma ratio. For example, considerable variability in bioavailability has been reported in healthy adults, with values ranging from 0.01 to 0.3[35].

Following melatonin ingestion, melatonin saliva and plasma concentrations are closely correlated[34], and orally administered melatonin emerges in the serum and saliva with nearly parallel time courses[33]. Assuming that the average saliva-to-plasma ratio is 0.37 after the administration of melatonin (based on previous research[33]), the mean estimated maximum plasma concentration after administration of Circadin 2 mg mini-tablets in children with ASD may actually be higher than the reported maximum plasma concentration in healthy adult volunteers with similar exposure (based on the estimated AUC in plasma). After administration of Circadin 2 mg tablets, the average C_{max} plasma value in adults was 483-1000 pg/ml. However, these results are difficult to interpret, as the groups varied in both age and gender, factors that can affect melatonin kinetics. For example, women generally have a C_{max} value that is four-fold higher than men. If exogenous melatonin appears in the plasma with the same time course as in saliva, the maximum concentration in the plasma after taking Circadin 2 mg mini-tablets will be reached earlier in children with ASD than in healthy adults after taking Circadin 2 mg tablets. This difference could be due to a faster absorption rate for mini-tablets compared to conventional sustained-release tablets (due to the larger surface area of the mini-tablets). In healthy adults, a half-life of 6 hours has been reported after taking Circadin 2 mg tablets. In our study, the median apparent terminal half-life was approximately 4 to 5 hours, which may suggest that children – or children with ASD – metabolize melatonin more rapidly than adults, or the volume of distribution may be smaller in children than in adults. To date, relatively little research has been performed regarding melatonin metabolism in children with ASD. A study in which serum melatonin and urine 6-SMT were measured following an intravenous infusion of melatonin in prepubescent, pubescent, and adult subjects[32] found that prepubescent children metabolize melatonin more rapidly than adults, and a second study found that melatonin metabolism may be delayed in children with Asperger syndrome[36]. In our study, because the group size was relatively small, it was not possible to perform subgroup analyses in order to compare PK between pubescent and prepubescent children. Following morning administration of Circadin, most subjects were assessed by the Observer's Assessment of Alertness/Sedation Scale to have a mild increase in sedation, with most scores indicating 'drowsy/normal speech' or 'slow reaction to verbal'. Sedation after the 2 mg Circadin dose peaked at (and after) 2 hours, which is around the T_{max} time of the PK profile, demonstrating PK/PD correlation.

Saliva melatonin concentrations at baseline were characteristic of daytime sampling, being low in the morning and increased over 12 hours towards the evening yet showed overall low endogenous levels. These results were confirmed by baseline 6-SMT measured in urine. This confirms the low levels found in earlier studies in children with ASD. Children with ASD can have an abnormal circadian melatonin profile, including lower nighttime melatonin levels[15,37-39]. Because nearly half of our subjects had used melatonin prior to the study, the study population may have been biased towards subject with lower baseline melatonin levels. However, because exogenous melatonin does not appear to affect the production of endogenous melatonin in terms of amplitude[40], and because endogenous melatonin was not suppressed by the administration of Circadin tablets in previous studies (our unpublished data), it is unlikely that these subjects had low baseline endogenous levels due to negative feedback from taking exogenous melatonin prior to the study. On the other hand, endogenous melatonin profiles may be age-specific or disorder-specific. For example, healthy children show a progressive decline from pre-school age in both nocturnal serum melatonin[31,41-43] and urinary metabolite excretion rate[44], suggesting that circadian rhythm decreases with

sexual maturation[31], causing a small increase in melatonin levels in pubescent children[45]. The putative effect of age on endogenous melatonin levels has been investigated only rarely in subjects with ASD. One recent study found no correlation between age and melatonin levels in children with Asperger syndrome[38], suggesting that melatonin levels are suppressed in early childhood in this population[38]; however, this apparent lack of relationship could also be attributed to other factors, including small sample size.

Although the administration of Circadin led to supraphysiologic melatonin levels, treatment was generally were tolerated, with no severe, serious or significant adverse events. Reported adverse events were consistent with known side effects[46,47]. The most frequent adverse events such as fatigue, somnolence and onset of sleep were to be expected based on melatonin's mechanism of action.

In conclusion, this study demonstrates the short-term safety, acceptability, and prolonged-release profile of Circadin mini-tablets in school-age children and adolescents with ASD. Importantly, the potential applications for this new, flexible formulation are not limited to children with neurodevelopmental disorders, but could also include other populations with sleep problems, including children and adolescents with ADHD, adult patients with a neurological disorder leading to dysphagia, and geriatric patients; however, the feasibility of giving mini-tablets to patients with difficulty swallowing should be determined. This initial study will be followed by a randomized, double-blind, placebo-controlled study designed to investigate the efficacy and long-term safety of Circadin mini-tablets in children and adolescents with neurodevelopmental disorders.

REFERENCES

1 Miano S, Ferri R. Epidemiology and management of insomnia in children with autistic spectrum disorders. *Paediatr Drugs.* 2010;12:75-84.
2 Richdale AL, Schreck KA. Sleep problems in autism spectrum disorders: prevalence, nature, & possible biopsychosocial aetiologies. *Sleep Med Rev.* 2009;13:403-411.
3 Gail WP, Sears LL, Allard A. Sleep problems in children with autism. *J Sleep Res.* 2004;13:265-268.
4 Patzold LM, Richdale AL, Tonge BJ. An investigation into sleep characteristics of children with autism and Asperger's Disorder. *J Paediatr Child Health.* 1998;34:528-533.
5 Richdale AL, Prior MR. The sleep/wake rhythm in children with autism. *Eur Child Adolesc Psychiatry.* 1995;4:175-186.
6 Wiggs L, Stores G. Sleep patterns and sleep disorders in children with autistic spectrum disorders: insights using parent report and actigraphy. *Dev Med Child Neurol.* 2004;46:372-380.
7 Meijer AM, Habekothe HT, Van Den Wittenboer GL. Time in bed, quality of sleep and school functioning of children. *J Sleep Res.* 2000;9:145-153.
8 Bourgeron T. The possible interplay of synaptic and clock genes in autism spectrum disorders. *Cold Spring Harb Symp Quant Biol.* 2007;72:645-654.
9 Chen F, Lemonnier E, Lazartigues A, Planche P. Sleep problems and information processing, a „disconnection effect' in autism? *Med Hypotheses.* 2006;66:1245-1246.
10 Jan JE, O'Donnell ME. Use of melatonin in the treatment of paediatric sleep disorders. *J Pineal Res.* 1996;21:193-199.
11 Smith AC, Dykens E, Greenberg F. Sleep disturbance in Smith-Magenis syndrome (del 17 p11.2). *Am J Med Genet.* 1998;81:186-191.
12 Didden R, Korzilius H, Smits MG, Curfs LM. Sleep problems in individuals with Angelman syndrome. *Am J Ment Retard.* 2004;109:275-284.
13 Wasdell MB, Jan JE, Bomben MM, Freeman RD, Rietveld WJ, Tai J, et al. A randomized, placebo-controlled trial of controlled release melatonin treatment of delayed sleep phase syndrome and impaired sleep maintenance in children with neurodevelopmental disabilities. *J Pineal Res.* 2008;44:57-64.
14 Doyen C, Mighiu D, Kaye K, Colineaux C, Beaumanoir C, Mouraeff Y, et al. Melatonin in children with autistic spectrum disorders: recent and practical data. *Eur Child Adolesc Psychiatry.* 2011;20:231-239.
15 Miyamoto A, Oki J, Takahashi S, Okuno A. Serum melatonin kinetics and long-term melatonin treatment for sleep disorders in Rett syndrome. *Brain Dev.* 1999;21:59-62.
16 Alvarez B, Dahlitz MJ, Vignau J, Parkes JD. The delayed sleep phase syndrome: clinical and investigative findings in 14 subjects. *J Neurol Neurosurg Psychiatry.* 1992;55:665-670.
17 Rossignol DA. Novel and emerging treatments for autism spectrum disorders: a systematic review. *Ann Clin Psychiatry.* 2009;21:213-236.
18 Garfinkel D, Laudon M, Zisapel N. Improvement of sleep quality by controlled-release melatonin in benzodiazepine-treated elderly insomniacs. *Arch Gerontol Geriatr.* 1997;24:223-231.
19 Jan JE, Hamilton D, Seward N, Fast DK, Freeman RD, Laudon M. Clinical trials of controlled-release melatonin in children with sleep-wake cycle disorders. *J Pineal Res.* 2000;29:34-39.
20 Jan JE, Connolly MB, Hamilton D, Freeman RD, Laudon M. Melatonin treatment of non-epileptic myoclonus in children. *Dev Med Child Neurol.* 1999;41:255-259.
21 De LH, Zisapel N, Laudon M. Prolonged-release melatonin for children with

neurodevelopmental disorders. *Pediatr Neurol.* 2011;45:23-26.
22 Shah T, Tse A, Gill H, Wong I, Sutcliffe A, Gringras P, et al. Administration of melatonin mixed with soft food and liquids for children with neurodevelopmental difficulties. *Dev Med Child Neurol.* 2008;50:845-849.
23 Spomer N, Klingmann V, Stoltenberg I, Lerch C, Meissner T, Breitkreutz J. Acceptance of uncoated mini-tablets in young children: results from a prospective exploratory cross-over study. *Arch Dis Child.* 2012;97:283-286.
24 Deacon S, Arendt J. Posture influences melatonin concentrations in plasma and saliva in humans. *Neurosci Lett.* 1994;167:191-194.
25 Chernik DA, Gillings D, Laine H, Hendler J, Silver JM, Davidson AB, et al. Validity and reliability of the Observer's Assessment of Alertness/Sedation Scale: study with intravenous midazolam. *J Clin Psychopharmacol.* 1990;10:244-251.
26 Lamas A, Lopez-Herce J. Monitoring sedation in the critically ill child. *Anaesthesia.* 2010;65:516-524.
27 Ernest TB, Elder DP, Martini LG, Roberts M, Ford JL. Developing paediatric medicines: identifying the needs and recognizing the challenges. *J Pharm Pharmacol.* 2007;59:1043-1055.
28 Thomson SA, Tuleu C, Wong IC, Keady S, Pitt KG, Sutcliffe AG. Minitablets: new modality to deliver medicines to preschool-aged children. *Pediatrics.* 2009;123:e235-e238.
29 Appleton RE, Gringras P, MENDS (Medicines for Children CTUUOLSG. Mends: the use of melatonin in children with neuro-developmental disorders and impaired sleep - a randomised, double-blind, placebo-controlled, parallel trial. *Arch Dis Child.* 2011;96:A1-A100.
30 Haimov I, Lavie P, Laudon M, Herer P, Vigder C, Zisapel N. Melatonin replacement therapy of elderly insomniacs. *Sleep.* 1995;18:598-603.
31 Waldhauser F, Boepple PA, Schemper M, Mansfield MJ, Crowley WF, Jr. Serum melatonin in central precocious puberty is lower than in age-matched prepubertal children. *J Clin Endocrinol Metab.* 1991;73:793-796.
32 Cavallo A, Ritschel WA. Pharmacokinetics of melatonin in human sexual maturation. *J Clin Endocrinol Metab.* 1996;81:1882-1886.
33 Vakkuri O, Leppaluoto J, Kauppila A. Oral administration and distribution of melatonin in human serum, saliva and urine. *Life Sci.* 1985;37:489-495.
34 Shirakawa S, Tsuchiya S, Tsutsumi Y, Kotorii T, Uchimura N, Sakamoto T, et al. Time course of saliva and serum melatonin levels after ingestion of melatonin. *Psychiatry Clin Neurosci.* 1998;52:266-267.
35 Fourtillan JB, Brisson AM, Gobin P, Ingrand I, Decourt JP, Girault J. Bioavailability of melatonin in humans after day-time administration of D(7) melatonin. *Biopharm Drug Dispos.* 2000;21:15-22.
36 Braam W, Didden R, Smits MG, Curfs LM. Melatonin for chronic insomnia in Angelman syndrome: a randomized placebo-controlled trial. *J Child Neurol.* 2008;23:649-654.
37 Zhdanova IV, Wurtman RJ, Wagstaff J. Effects of a low dose of melatonin on sleep in children with Angelman syndrome. *J Pediatr Endocrinol Metab.* 1999;12:57-67.
38 Takaesu Y, Komada Y, Inoue Y. Melatonin profile and its relation to circadian rhythm sleep disorders in Angelman syndrome patients. *Sleep Med.* 2012.
39 Jan JE, Freeman RD, Fast DK. Melatonin treatment of sleep-wake cycle disorders in children and adolescents. *Dev Med Child Neurol.* 1999;41:491-500.
40 Matsumoto M, Sack RL, Blood ML, Lewy AJ. The amplitude of endogenous melatonin production is not affected by melatonin treatment in humans. *J Pineal Res.* 1997;22:42-44.
41 Salti R, Galluzzi F, Bindi G, Perfetto F, Tarquini R, Halberg F, et al. Nocturnal melatonin patterns in children. *J Clin Endocrinol Metab.* 2000;85:2137-2144.
42 Cavallo A. Plasma melatonin rhythm in normal puberty: interactions of age and pubertal stages. *Neuroendocrinology.* 1992;55:372-379.
43 Cavallo A. Melatonin secretion during adrenarche in normal human puberty and in pubertal disorders. *J Pineal Res.* 1992;12:71-78.
44 Cavallo A, Dolan LM. 6-Hydroxymelatonin sulfate excretion in human puberty. *J Pineal Res.* 1996;21:225-230.
45 Molina CA, Munoz HA, Uberos FJ, Acuna CD, Molina Font JA. [Pineal functioning (melatonin levels) in healthy children of different ages. An update and the value of pineal gland study in pediatrics]. *An Esp Pediatr.* 1996;45:33-44.
46 Pediatric Formulary („Kinderformularium'). 2014.
47 Andersen IM, Kaczmarska J, McGrew SG, Malow BA. Melatonin for insomnia in children with autism spectrum disorders. *J Child Neurol.* 2008;23:482-485.

TABLE 1 **Summary PK parameters of melatonin per treatment**

Treatment	N	C_{max} (pg/ml)	t_{max} (h)	$^{a}AUC_{0\text{-last}}$ (pg.h/ml)	$^{b}AUC_{0\text{-}\infty}$ (pg.h/ml)	$t_{1/2}$ (h)
Circadin 2 mg	14	965 (1,170)	1.57 (0.762)	2,370 (1,240)	2,420 (1,100)	5.74 (3.31)
Circadin 2 mg*	11	410 (210)	1.73 (0.792)	1,960 (1,030)	2,150 (960)	4.87 (1.87)
Circadin 10 mg	14	3,970 (2,830)	1.37 (0.640)	12,300 (7,830)	13,00 (7,680)	4.44 (1.69)

Summary of melatonin pharmacokinetic parameters (mean and SD) in the PK population. Data from subject 9 and 10 were excluded from analysis as these subjects were dosed with a melatonin formulation that erroneously did not comply with the desired in vitro release profile. *sub-analysis PK population. **a.** Area under the saliva concentration-time curve from the first to the last observation; **b.** Area under the saliva concentration-time curve from the first observation to infinity.

FIGURE 1 **Graphs showing simulated concentration-time profiles in plasma (black line) after 2-mg morning dose (upper left panel), 10-mg morning dose (upper right panel), 2-mg evening dose (lower left panel) and 10-mg evening dose (lower right panel) administered to a child of 35 kg body weight in fed condition.** In the current study, a morning dose was proposed. An allometric scaling approach was used, assuming dose linearity from 2 to 10 mg, a smaller distribution volume (25 L instead of 50 L and higher metabolism rate (half-life of 0.42 hr) in a healthy child compared to an average adult. Concentration-time profile of endogenous melatonin concentrations are shown in black and was fitted based on data from healthy children. The baseline profile (represented by grey line) in the study population can be different from this profile. Sampling time points after Circadin administration are indicated by vertical black lines.

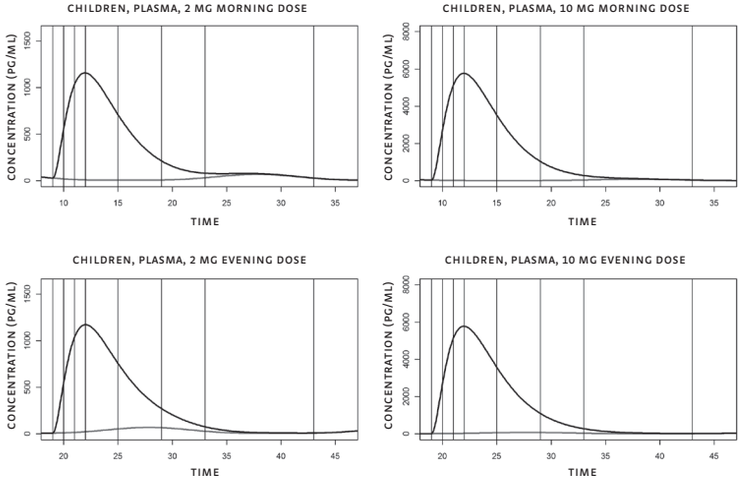


FIGURE 2 Individual concentration-time profile of melatonin measured in saliva on a semi-log scale. The continuous line represents the measured concentrations after 2 mg Circadin mini-tablets; the dashed line the concentrations after 10 mg Circadin mini-tablets. The assay's lower limit of quantification (2 pg/ml) is plotted as a horizontal dashed line.

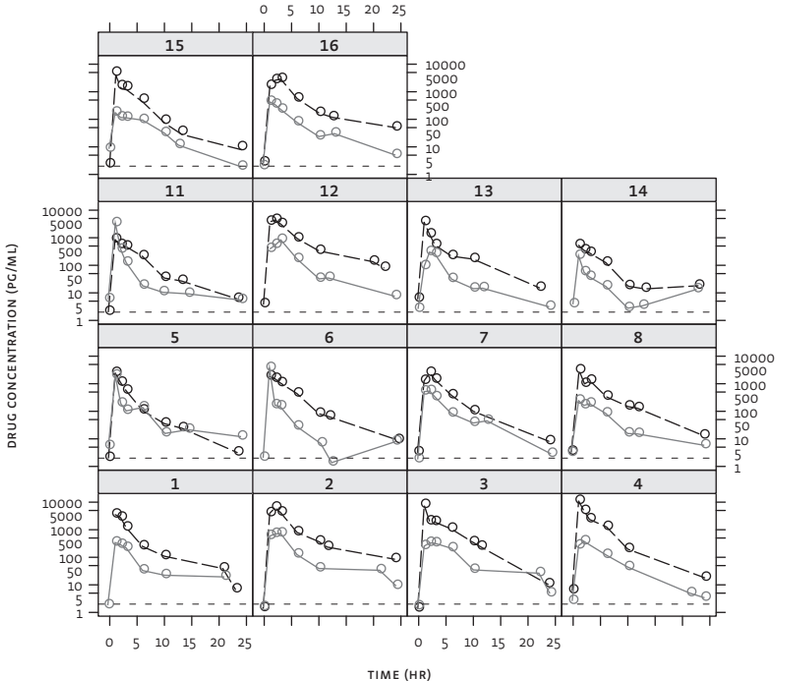


FIGURE 3 Concentration-time profile of melatonin measured in saliva.

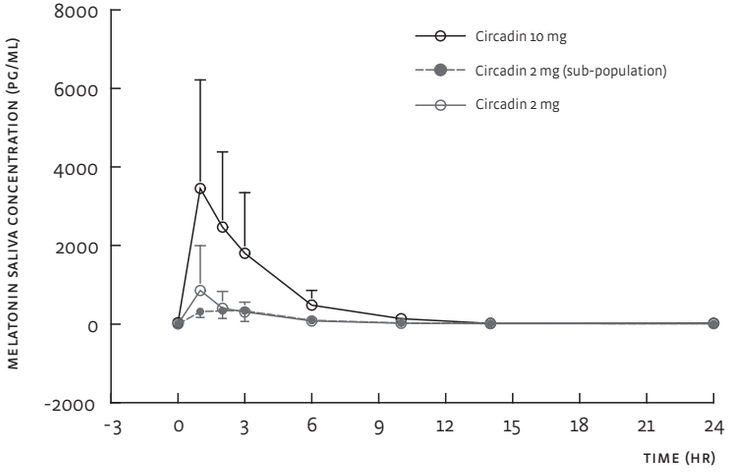
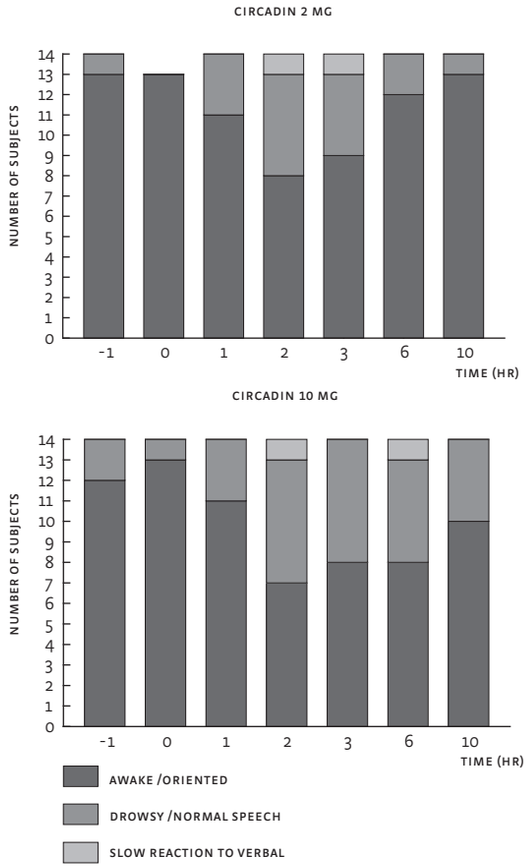


FIGURE 4 Observer's Assessment of Alertness/Sedation Scale scores before and after administration of 2 mg Circadin or 10 mg Circadin, PK population. Number of subjects in PK population with score 'awake/oriented', 'slow reaction to verbal, 'drowsy/normal speech' are presented per time point. None of the subjects scored 'reacts to soft touch'.



CHAPTER 9

Pharmacokinetics and pharmacodynamics of a new highly concentrated intranasal midazolam formulation for conscious sedation

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ABSTRACT

Background: To evaluate the pharmacokinetics, safety, nasal tolerance and effects on sedation of a highly concentrated aqueous intranasal midazolam formulation (Nazolam) and to compare these to intravenous midazolam.Methods: In this four-way crossover, double-blind, double-dummy, randomized, placebo-controlled study, 16 subjects received 2.5 mg Nazolam, 5.0 mg Nazolam, 2.5 mg intravenous midazolam or placebo on different occasions. Pharmacokinetics of midazolam and α-hydroxy-midazolam were characterized and related to outcome variables for sedation (Saccadic Peak Velocity, the Bond and Lader Visual Analogue Scale for sedation, the Simple Reaction Time Task and the Observer's Assessment of Alertness/Sedation). Nasal tolerance was evaluated through subject reporting and ENT examination.
Results: Nazolam bio-availability was 75%. Maximal plasma concentrations of 31 ng/ml (CV, 42.3%) were reached after 11 minutes (2.5 mg Nazolam), and of 66 ng/ml (CV, 31.5%) after 14 minutes (5.0 mg Nazolam). Nazolam displayed a significant effect on OAA/S scores. Sedation onset (based on SPV change) occurred 1 minute after administration of 2.5 mg intravenous midazolam, 7 minutes after 2.5 mg Nazolam, and 4 minutes after 5 mg Nazolam. Sedation duration was 85 minutes for 2.5 mg intravenous midazolam, 47 minutes for 2.5 mg Nazolam, and 106 minutes for 5.0 mg Nazolam. Nazolam did not lead to nasal mucosa damage.
Conclusions: This study demonstrates the nasal tolerance, safety and efficacy of Nazolam. When considering the preparation time needed for obtaining venous access, conscious sedation can be achieved in the same time span as needed for intravenous midazolam. Nazolam may offer important advantages in conscious sedation and epilepsy.

Introduction

Midazolam is a short acting benzodiazepine with anxiolytic, sedative, anticonvulsant and skeletal muscle relaxant properties. Due to its fast onset and recovery profile, it is the preferred medication for obtaining conscious sedation and management of epileptic seizures. Midazolam is used in a wide range of indications for conscious sedation, including sedation for the majority of outpatient diagnostic, therapeutic and endoscopic procedures and sedation for the preparation of general anesthesia in hospitalized patients. Administration of midazolam is generally intravenous as other administration routes such as oral, rectal, subcutaneous and buccal lead to a delayed onset of efficacy and to a large inter-individual variability in efficacy onset. The nasal route therefore appears to be a very convenient route of administration for conscious sedation and for the lay treatment of acute epileptic seizures. As conscious sedation is usually applied in time critical or logistically optimized hospital settings, intravenous administration is preferred over these other administration routes despite the disadvantages of requiring intravenous access and the intermittent dosing protocol necessary to avoid high initial peak midazolam plasma concentrations.

Nasal administration of midazolam is a simple, useful and reliable alternative to the parenteral route. It offers several practical advantages, as it allows for direct, easy and needle free administration, and can be safely administered without the need for professional assistance. Needle free sedation is particularly advantageous in children, patients with needle phobia, patients with varicose (difficult accessible) veins uncooperative patients and in emergency room settings, in dentistry and other non-hospital settings. In addition, it provides potential for rapid systemic drug absorption and quick onset of action without initial high peak concentrations, which may lead to respiratory depression.

Unfortunately, nasal delivery of midazolam has not been very successful until now due to the absence of solvents that are able to dissolve midazolam at efficacious dosages without leading to nasal mucosa damage. To avoid swallowing and gastrointestinal absorption of excess fluid reaching the oropharynx, the maximal volume of nasal application is ideally restricted to approximately 100 μL^1, requiring the efficacious dose of midazolam to be dissolved within this volume. Higher volumes lead to nasal drop-off or swallowing, which in turn may lead to lower and unpredictable concentrations, and a relatively long onset of action. This in turn can cause overdosing, if a second dose is applied because the first one did not act fast enough. Therefore, highly concentrated solutions with a high bioavailability are essential to achieve clinically relevant plasma concentrations after nasal application. Attempts to overcome this limitation by formulating midazolam in organic solvents or absorption enhancers that allow for dosing volumes as low as 100 µl have largely failed due to the fact that these solvents are typically irritating to the highly sensitive and easily disrupted nasal mucosa tissue.

Recently, a highly concentrated, aqueous midazolam formulation (Nazolam) that allows dosing of 100 µl or below has become available. Because of the aqueous nature of the midazolam formulation, nasal tolerance was expected to be good. If proven successful, this aqueous midazolam administration will be the first to address all limitations of currently intravenous and intranasal applications of midazolam. In this study, the pharmacokinetics (PK), efficacy and tolerability of this nasal midazolam formulation were evaluated and compared to intravenous midazolam.

Methods

Study design

This was a randomized, double-blind, double-dummy placebo-controlled, four-way crossover study in 16 healthy volunteers. The study was conducted in adherence to the guidelines of the International Conference on Harmonisation Guideline for Good Clinical Practice and in accordance with the principles of the Declaration of Helsinki. The study was performed at the Centre for Human

Drug Research in Leiden, the Netherlands, and approved by the local ethics committee of Leiden University Medical Center (Leiden) (ref: P10.215) and registered with EudraCT (ref: 2010-023425-38). The subjects consented in writing to the study after full explanation of what was involved.

Subjects

Inclusion criteria for this study were for healthy male or female volunteers aged 18-55 years, with a body mass index of 18-33 kg/m^2. All subjects had to be willing and able to comply with study procedures. Exclusion criteria included history of central nervous system or psychiatric disease, history of drug, substance and/or alcohol abuse, and abnormal findings on screening medical history, physical examination, ECG, vital signs and/or blood and urine laboratory profile. Subjects with anatomical anomalies causing obstruction of the nares, recent (< 4 weeks) nose bleeds, or with a history of chronic nasal obstruction or clinically significant nasal surgery that could affect absorption of or tolerance to midazolam were excluded. Subjects with clinically significant upper respiratory infection, common cold or flu-like symptoms and/or rhinitis at screening were also excluded. Subjects were not allowed to use any medication which could affect the metabolism of midazolam or the performance of CNS measurements from 2 weeks prior to the start of the study days. Subjects were not allowed to consume more than 8 units of xanthine-containing products per day. Subjects had to refrain from consumption of xanthine- or alcohol-containing products and smoking from 1 day prior to admission until the end of the study day. On study days, intake of medication, alcohol, or drugs was questioned and a urine drug screen and pregnancy test and an alcohol breath test were performed before any study-related procedures were started.

Study treatments

Study treatments were administered as a unit-dose nasal spray containing 2.5 or 5.0 mg midazolam (Nazolam) or the same formulation without midazolam, and intravenous solution containing 2.5 mg midazolam or saline. On each study day, subjects received both an intranasal and intravenous study drug administration. The subjects on each study occasion received one of four treatments: (i) 2.5 mg intranasal midazolam and intravenous placebo; (ii) 5 mg intranasal midazolam and intravenous placebo; (iii) intranasal placebo and 2.5 mg intravenous midazolam, and (iv) intranasal placebo and intravenous placebo. The sequence of treatments was randomized, defined by a William Square Design, and study days were separated by washout periods of at least 6 days. Doses were administered in the non-fasted state.
The recommended starting dose for conscious sedation is 2-2.5 mg[2]. The lowest dose administered intranasally was therefore 2.5 mg, based on the assumption of a high bioavailability. In addition, a 5-mg dose was tested because this dose is relevant in the treatment of epilepsy. Moreover, by studying both dosages, an indication of dose proportionality and dose-response relationships of the intranasal formulation could be obtained. The concentration of the aqueous midazolam spray was 55.6 mg/ml midazolam HCl (50 mg midazolam base per ml) and the volume was 50 µl for the 2.5-mg dose and 100 µl for the 5-mg dose. The spray was administered in the same nostril throughout the study by the supervising physician.
The dosing regimen for the administration of 2.5 mg intravenous midazolam was 1 mg/30 seconds, which is in accordance with the SMPC of midazolam for the indication of conscious sedation.

Pharmacokinetic methods

Venous blood samples for pharmacokinetic analyses were obtained via an indwelling catheter before administration and at 1 minute and 15 seconds, and at every 3 minutes (until 30 minutes), every 10 minutes (until 60 minutes), every 30 minutes (until 2 hours), and at 3, 4, 6, 8 and 12 hours after drug administration. The Vacutainer tubes with lithium-heparin containing the blood samples were gently mixed by inversion (~8-10 times) and kept on wet ice thereafter. The samples were processed by centrifugation (10 minutes at 2-8

°C at 2000x*g*) within 30 minutes after the sample was drawn and the plasma was stored -80 °C until analysis. Bioanalytical analysis was performed by ABL (Analytisch Biochemisch Laboratorium BV, Assen, The Netherlands). Plasma concentrations of midazolam and its metabolite α-hydroxy-midazolam were determined using liquid chromatography coupled with tandem mass spectrometry. QC concentrations included QC-Low (target midazolam or α-hydroxy-midazolam concentrations in human heparin plasma of 0.300 ng/ml), QC-Medium (target concentration of 3.00 ng/ml) and QC-High (target concentration of 75.0 ng/ml). Assay specifics included acceptable precision (total CVs of 15% for all QC target concentrations), good accuracy (mean absolute biases values for all QC target concentrations of 15%) and adequate incurred sample reproducibility (difference of ≤ 20% for all reanalyzed midazolam samples and 98% of reanalyzed α-hydroxy-midazolam samples). The analytical range of the assay for both the parent and the metabolite was 0.100 – 100 ng/ml.

Pharmacodynamic methods

The 'Neurocart' is a battery of sensitive tests for a wide range of CNS domains that was developed to examine different kinds of CNS-active drugs including benzodiazepines[3-6]. All tests were performed twice at baseline, and repeated in the following order at the same time points as the PK blood sampling. Measurements were performed in a quiet room with ambient illumination with only one subject per session in the same room.

SACCADIC PEAK VELOCITY

Saccadic peak velocity is one of the most sensitive parameters for sedation[7-9] and was therefore used to evaluate the onset and duration of pharmacological effect of intranasal midazolam. The use of a computerized measurement of saccadic eye movements has been described elsewhere[9-11]. The definition of the onset and duration of pharmacological effect (sedation) was based on the subject's individual variability in SPV. Onset of sedation was defined as the (linearly interpolated) time point at which the SPV reached minus 2 standard deviations (SD) of the pre-value (baseline) SPV level for the occasion. Duration was defined as the total amount of time that the response was below the minus 2 SDs threshold; this total time could be made up of a number of episodes if the threshold was crossed repeatedly before complete termination of the effect.

VISUAL ANALOGUE SCALES

Visual analogue scales as originally described by others[12], have been previously used to quantify subjective effects of benzodiazepines[13] and to evaluate sedative effect of both intranasal and intravenous midazolam[14-26]. In this study, by using VAS Bond & Lader, the ‚directions' of different scales on a form were alternated, to avoid habitual scoring by subjects.

SIMPLE REACTION TIME TASK

The Simple Reaction Time Task measures the attention and speed of information processing of the subject. In this task, participants view a black computer screen. At random intervals (0.5 – 1.5 seconds), a white circle appears in the center of the computer screen. Subjects were instructed to press the space bar with the index finger of their dominant hand each time the circle appears. They were instructed to respond as quickly as possible after appearance of the circle. A total of 40 circles were presented, and the duration of the task was approximately 1 minute. The outcome of the task is the time between stimulus display and response. It has been shown to respond to several classes of sedative drugs[27]. Several previous studies also showed the positive application of SRTT in the investigation of midazolam's sedative effect[28-30]. The SRTT can be regarded as a clinically relevant measure that represents the level of dysfunctioning that may be caused by sedation and was included in this trial to evaluate whether recovery from sedation was similar for intravenous and intranasal dosing.

OBSERVER'S ASSESSMENT OF ALERTNESS/SEDATION

The Observer's Assessment of Alertness/Sedation Scale was previously developed to objectively measure the level of alertness in subjects who are sedated. The OAA/S Scale has been shown to be reliable and valid and to be sensitive to the level of midazolam administered [31]. The OAA/S has been used extensively in studies with intravenous midazolam[15,16,20,24–26,32].

Safety assessments

Safety assessments including adverse events (AE) monitoring, 12-lead ECGs, and laboratory safety tests were conducted at intervals throughout the study. Transcutaneous oxygen saturation levels were monitored during the first 6 hours after administration and blood pressure and heart rate until discharge (12 hours after administration).

Nasal tolerance assessments

Nasal safety was monitored by inspection of the nasal mucosa by a physician trained by an ENT medical specialist and by subject self assessment (subjective monitoring of congestion, irritation, pain, runniness and loss of smell). Nasal symptoms were assessed prior to and after nasal application and, if present, specified as (I) congestion or stuffiness; (II) irritation or itchiness; (III) runniness; (IV) pain or discomfort and/or (V) loss of or abnormal smell. In case of a nasal symptom, the severity was graded as (I) mild symptoms (minor awareness of symptoms, lasting up to an hour); (II) moderate symptoms (moderate awareness of symptoms, lasting up to 12 hours), or (III) severe symptoms (strong awareness of symptoms, lasting more than 12 hours). In addition, local nasal tolerance was assessed by means of a regular nasal examination using the following scoring system: (I) no visible abnormality; (II) mild abnormality (< 1 cm of erythema and/or swelling and/or other visible abnormality); (III) moderate abnormality (1-2 cm of erythema and/or swelling and/or other visible abnormality), or (IV) severe abnormality (> 2 cm of erythema and/or swelling and/or other visible abnormality). All observed or reported AEs were recorded for all subjects and AEs were classified as mild, moderate or severe and their relationship to study drug was assessed by the investigator.

Pharmacokinetic analysis

Pharmacokinetic parameters for midazolam and α-hydroxy-midazolam were estimated using non-compartmental modeling (WinNonlin 5.2; Pharsight, Mountain View, CA). The distributions of the dose-normalized PK parameters were compared using ANOVA.

Statistical methods

Sample size was determined based on a presumed onset of sedative effect of midazolam as defined based on a decrease in saccadic SPV of > 2SD from baseline. In a previous study performed by our research group the inter-subject CV of the time of onset defined on the basis of a decline in SPV was 62.5%. In this previous study the intra-subject CV could not be calculated, however, assuming the intra-subject CV to be smaller than the inter-subject CV, an intra-subject CV of 50% was used for sample size calculations. Sample size calculations were performed in nQuery (version 7.0). It was determined that a sample size of 16 would have 80% power to detect a difference in mean time of onset of sedative effect of 3.283 minutes, assuming a standard deviation of differences of 4.380, using a paired T-test with a 0.05 two-sided significance level.

SRTT and OAA/S data were log-transformed prior to analysis to correct for the expected log-normal distribution of the data and analysis was performed on log transformed data. Repeatedly measured pharmacodynamic data (SPV, VAS, SRTT, and OAA/S) were compared with a mixed model analysis of variance (using SAS PROC MIXED) with fixed factors treatment, period, time and treatment by time, random factors subject, subject by treatment and subject by time and the baseline value (average over all measurements at or before time=0) as covariate. The contrast between the midazolam treatments and

placebo were calculated within the statistical model. For onset of sedation based on SPV, the SD of the SPV during the whole placebo period was calculated for each subject, and the threshold of sedation was determined as the baseline value per period minus 2 SDs. Onset and duration of sedative effect were compared between treatment groups, assuming that the effect sizes of the different treatment groups were comparable. As OAA/S is a categorical variable, it was not assumed to be normally distributed. Therefore, an additional analysis was performed using the GLIMMIX procedure. In this procedure, all OAA/S data (including placebo) after dosing (time=0) followed a multinomial distribution and were compared with a mixed model analysis of variance with fixed factors treatment and period and random factors subject and subject by time. All calculations were performed using SAS for windows V9.1.3 (SAS Institute, Inc., Cary, NC, USA).

Results

Subjects

16 healthy subjects (8 male, 8 female) were enrolled in this study. They were on average 26 years old (range 19-53 years), and had an average body mass index of 23.2 kg/m^2 (range 19.6-28.1 kg/m^2). All subjects had negative pre-dose urine tests for drugs of abuse, including benzodiazepines. Concomitant medication used during the study period included paracetamol (up to 1.0 g per day), noscapine, acetylsalicylic acid, and xylometazoline (one subject, stopped more than 1 day before study drug administration). All subjects completed the study.

Pharmacokinetic results

The PK parameters and mean concentration-time profiles of midazolam and α-hydroxy-midazolam are shown in Table 1 and Figure 1. The intravenous data of 2 subjects had to be excluded due to sampling failures on 2 occasions (both 2.5 mg intravenous midazolam). In- or exclusion of the data gathered during these occasions did not significantly change the pharmacokinetic parameter estimates. Doubling of the intranasal midazolam dose resulted in dose-proportional increases in AUC and maximum concentration (AUC: mean increase, 2.0-fold; C_{max}: mean increase, 2.2-fold). Dose-normalized C_{max} and $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ were higher for the intravenous formulation than for the intranasal formulations. The overall concentration-time profiles of the intranasal formulations showed no second peak and the formation of metabolite was low and the relative amounts formed compared to the parent compound were comparable with the formation of metabolite after intravenous administration. Mean ratio of α-hydroxy-midazolam AUC to midazolam AUC after intranasal midazolam administration was 0.2 for all formulations and dosages.

The midazolam intravenous administration displayed a nine-fold ratio between the highest and lowest observed C_{max} value, whereas the ratio between the highest and lowest C_{max} for the intranasal 2.5 mg administration was seven-fold, and for the 5.0 mg intranasal administration three-fold, which led to the coefficients of variation (CV) in C_{max} included in Table 1.

Pharmacodynamic results

SACCADIC PEAK VELOCITY

A marked and time-dependent decrease in SPV was seen after midazolam administration until three hours after administration (Figure 2). There was a statistically significant difference in SPV between midazolam 2.5 mg intranasal and 5.0 mg intranasal ($p < 0.001$; 35.3, 95% CI = 20.6, 50.0).

Onset of action of midazolam (as defined by a decrease in SPV of more than 2SD below baseline) occurred 7±4.4 minutes after administration of midazolam 2.5 mg intranasal, 4±1.8 minutes after midazolam 5.0 mg intranasal. Onset of action after administration of midazolam 2.5 intravenous occurred on average 1±0.7 minutes after administration. There was a statistically significant difference between midazolam 2.5 mg intranasal and 2.5 mg intravenous ($p < 0.001$; 6.2, 95% CI = 4.2, 6,2), midazolam 5.0 mg intranasal and 2.5 mg

intravenous (p = 0.007, 2.7, 95% CI 0.8, 4.7) and midazolam 2.5 mg intranasal and 5.0 mg intranasal (p = 0.001, 3.5, 95% CI = 1.5, 5.4)

Duration of action as defined by a 2SD decrease in SPV was on average 76±80.4 minutes after administration of midazolam 2.5 mg intranasal and 145±104.9 minutes of midazolam 5.0 mg intranasal. Duration of action was on average 118±95.6 minutes after 2.5 mg intravenous midazolam. There was a statistically significant difference between 2.5 mg intranasal and intravenous midazolam (p = 0.03, -38.5, 95% CI -60.6, -3.9) and between the two intranasal dose levels (p = 0.001, -53.4, 95% CI -69.9, -28.0), but not between 2.5 mg intravenous and 5.0 mg intranasal.

VISUAL ANALOGUE SCALES

Subjective alertness decreased after administration in a time-dependent manner in all midazolam groups. There was a statistically significant difference between the intranasal dose levels (p = 0.0009, 1.9, 95% CI 0.8, 2.9; see Figure 3). There were no effects on VAS Calmness or VAS Mood.

SIMPLE REACTION TIME TASK

Midazolam had a marked effect on the reaction time with a statistically significant difference between the intranasal dose levels (p = 0.0005, -10/8, 95% CI -16.2, -5.2; see Figure 4).

OBSERVER'S ASSESSMENT OF ALERTNESS/SEDATION

Intranasal midazolam displayed a significant effect on sedation as measured using OAA/S. Levels of sedation after midazolam intranasal 2.5 mg and midazolam intravenous 2.5 mg administration were comparable, whereas midazolam intranasal 5.0 mg led to higher sedation levels (see Figure 5). The odds ratio (defined as the chance (odds) that a subject scored an OAA/S of 1 (awake/orientated, indicating no sedation) during one treatment versus the other) was 2.3 (p < 0.0001; 95% CI = 1.63, 3.18) for the contrast midazolam 2.5 mg intranasal vs midazolam 2.5 mg intravenous, 0.8 (p = 0.30; 95% CI = 0.62, 1.16) for the contrast midazolam 5.0 mg intranasal vs midazolam 2.5 mg intravenous, and 2.7 (p < 0.0001; 95% CI = 1.92-3.75) for the contrast midazolam 2.5 mg intranasal vs midazolam 5.0 mg intranasal.

Safety

GENERAL

There were few adverse events. Adverse events were mild en transient and equally distributed over the 2.5 mg (17) and 5.0 mg intranasal (22) and intravenous (15) groups. The most common adverse events were somnolence and headache, which were reported 46 and 13 times in total and in 31-94% (somnolence) and 6-25% (headache) of subjects (including placebo) and reported in all treatment groups. Administration of a single dose midazolam did not result in clinically significant changes in physical findings or ECG recordings.

In general, types of adverse events for the intranasal and intravenous formulations of 2.5 mg midazolam were similar. There were more cases of diplopia in the intranasal treatment groups (1 case in 2.5 mg group and 6 cases in 5 mg group), which could be explained by midazolam's characteristic (dose-related) benzodiazepine effects on $GABA_A$-receptors in the central nervous system. Since $GABA_A$-receptors do not occur peripherally, it is unlikely that this is due to local effects of the intranasal formulation. The larger number of cases of sleep-related symptoms in the 5.0 mg intranasal midazolam group likely results from the higher AUC in this treatment group. There were more cases of attention disturbances in the 2.5 mg intranasal midazolam treatment group compared to the 5.0 mg intranasal and 2.5 mg intravenous midazolam groups. Attention disturbance represents the lower end of the spectrum of $GABA_A$ -effects, and subjects in the low-dose intranasal treatment group may have not been sedated to such a level that somnolence occurred, but enough to experience attention problems.

NASAL

No significant abnormalities were found during nasal examination in any of the subjects. Mild and transient visible abnormalities (< 1 cm) were observed

for one subject almost 12 hours after administration of 2.5 mg intranasal midazolam and for one subject 2 hours after administration of 5.0 mg intranasal midazolam, which all resolved spontaneously. Mild nasal symptoms were observed in 2 subjects 1 hour after administration of 5.0 mg intranasal midazolam, which resolved within 1-2 hours. In one subject these symptoms may be related to mild runniness already observed before dosing. One subject reported rhinorrhea, and one reported sneezing after 2.5 mg intranasal midazolam. After 5.0 mg intranasal midazolam, one subject reported cough, one reported irritation, one reported epistaxis (at the day after administration, where during the study day no nasal symptoms or visible abnormalities were observed), and two reported sneezing.

OXYGEN SATURATION

No clinically relevant decreases in transcutaneous oxygen saturation or blood pressure were observed.

Discussion

This study is the first to report on the pharmacokinetics and effects on sedation of Nazolam. Nazolam is a new aqueous nasal formulation of midazolam that does not lead to nasal tissue damage and delivers small enough volumes to be fully absorbed by the nasal mucosa, yet containing sufficiently high concentrations of midazolam to establish clinically relevant systemic midazolam concentrations. For all midazolam treatment groups, effects were seen on pharmacodynamic outcome variables of sedation and clinically relevant levels of sedation as measured using OAA/S (≥ score 2, or drowsy/normal speech) were achieved within minutes after administration. It is therefore clear that use of Nazolam is an effective, convenient and safe method of inducing conscious sedation for a wide range of applications.

Most previous studies used formulations that led to nasal run-off or nasal mucosal damage, or were hampered by flaws in the experimental design. Single administration of Nazolam, however, was well tolerated and safe in healthy adult subjects. Nasal symptoms were mild and transient and only observed in two patients. Furthermore, the aqueous nature of the formulation allows for a pH driven transport into the buffered nasal mucosa constitution allowing for a fast onset of efficacy of midazolam in a similar time window as observed after intravenous administration of midazolam. Mean absolute bioavailability of Nazolam was high (approximately 75%) and clinically effective concentrations were reached within minutes after nasal administration. Observed maximal systemic midazolam concentrations were comparable to those observed after oral midazolam administration[33,34]. Lower (and thus more favorable) and less variable peak concentrations were seen after intranasal compared to intravenous administration of midazolam. Nazolam showed dose proportional pharmacokinetics in the investigated dose range (2.5 – 5.0 mg). Several pharmacokinetic studies have been published using intranasal formulations[35-42]. Although some showed comparable pharmacokinetic results, different nasal formulations were used, mainly using very large volumes, or high concentrations of organic solvents or absorption enhancers. There were no signs of important contribution of ingestion related intestinal absorption, as the overall concentration-time profiles of intranasal formulations did not show a second peak and the formation of metabolite was low and comparable with intravenous levels. The absence of clinically relevant decreases in transcutaneous oxygen saturation parameters and blood pressure in this study indicate that the safety profile of nasal midazolam is comparable to that observed after oral midazolam administration.

Saccadic peak velocity is generally considered as a sensitive and reproducible biomarker for the sedative effects of benzodiazepines[43] and was therefore used as a biomarker of pharmacological effect of midazolam in this study. SPV has already been used as an outcome variable in several previous studies with intravenous midazolam[44-51] and changes in saccadic eye movements allow the accurate recognition of the wake-sleep transition[8,52]. In this study, an attempt was made to compare the onset and duration of pharmacological effect of the different midazolam formulations as accurately and realistically

as possible. In a recent review on biomarkers for the effects of benzodiazepines in healthy subjects, a relationship between SPV reduction and clinical efficacy was described, as all reviewed benzodiazepines caused an impairment of saccadic peak velocity, which was closely related to the therapeutic dose[43]. Therefore, SPV was used in this study to evaluate the onset and duration of pharmacological effect of intranasal midazolam. The definition of the onset and duration of pharmacological effect (sedation) was based on the subject's individual variability in SPV under placebo. As expected, intranasal midazolam led to a marked decrease in saccadic peak velocity at both investigated doses. Nazolam 2.5 mg led to conscious sedation in all individuals as reflected in the clear SPV decline observed for all subjects, showing that SPV is a sensitive biomarker and a good choice for a proof-of-pharmacology study such as the current one.

The effects of intranasal midazolam on SPV and subjective VAS alertness increased in a dose proportional fashion. The time effect curves of SPV and VAS alertness were comparable, which supports the appropriateness of the use of SPV as a surrogate marker for the sedative effect of midazolam. However, SPV was clearly more sensitive to midazolam effects than VAS alertness, as the observed effects of midazolam on SPV started earlier and returned to baseline later than those on VAS alertness. The use of SPV to define onset of pharmacological effect is therefore supported by the current data. Onset and duration of sedation were compared between treatment groups. The duration of sedation was slightly shorter for intranasal than for the intravenous formulation, but this was not statistically significant.

No effects were seen on subjective mood and calmness (as assessed by VAS), but this was not unexpected as it is in accordance with our experience with studies in healthy non-anxious subjects and likely related to a floor effect in the assessment of subjective calmness.

To assess the impact of ongoing sedation due to midazolam on normal functioning, the effect on the simple reaction time task (SRTT) was assessed. Reaction time was increased by midazolam for about as long as the other pharmacodynamic effects (slowing of SPV and decrease in VAS alertness). The effects on SSRT of both the intranasal and the intravenous formulation returned to baseline in almost 2 hours for all formulations.

In conclusion, this study demonstrates that clinically effective concentrations can be reached within minutes after nasal application of a highly concentrated midazolam formulation with sedation profiles comparable to those observed after intravenous midazolam administration. When considering the preparation time needed for obtaining venous access, conscious sedation can be achieved in the same time span for nasal as for intravenous administration of midazolam. Potential applications of this new formulation are not limited to settings where midazolam is currently being used intravenously, but could also include settings in which intravenous access is not feasible such as in children and patients with needle phobia, uncooperative patients and in urgent / emergency room situations. With the demonstrated absence of initial high peak plasma concentrations, nasal delivery also allows for safe and efficacious conscious sedation out-side hospital settings such as the general practitioner office and dentistry settings. Finally, as the absorption capacity of the nasal mucosa is limited to 100 µl per nostril, nasal administration is relatively safe to overdosing. The immediate and non-invasive characteristics of this new formulation offer important advantages for clinical use in conscious sedation and in epilepsy.

REFERENCES

1 Marx D, Birkhoff M. Multi-dose container for nasal and ophthalmic drugs: a preservative free future? In: Rundfeldt C, editor. Drug Development - A case study based insight into modern strategies.Rijeka, Croatia: InTech; 2011. 509-524.

2 SmPC Midazolam (Dormicum) 5mg/mL. 1-17. 2013.

3 de Haas SL, de Visser SJ, van der Post JP, de SM, Schoemaker RC, Rijnbeek B, et al. Pharmacodynamic and pharmacokinetic effects of TPA023, a GABA(A) alpha(2,3) subtype-selective agonist, compared to lorazepam and placebo in healthy volunteers. *J Psychopharmacol.* 2007;21:374-383.

4 van Steveninck AL, Gieschke R, Schoemaker HC, Pieters MS, Kroon JM, Breimer DD, et al. Pharmacodynamic interactions of diazepam and intravenous alcohol at pseudo steady state. *Psychopharmacology (Berl).* 1993;110:471-478.

5 van Steveninck AL, Gieschke R, Schoemaker RC, Roncari G, Tuk B, Pieters MS, et al. Pharmacokinetic and pharmacodynamic interactions of bretazenil and diazepam with alcohol. *Br J Clin Pharmacol.* 1996;41:565-573.

6 Zoethout RW, Schoemaker RC, Zuurman L, van PH, Dahan A, Cohen AF, et al. Central nervous system effects of alcohol at a pseudo-steady-state concentration using alcohol clamping in healthy volunteers. *Br J Clin Pharmacol.* 2009;68:524-534.

7 Van Steveninck AL, Schoemaker HC, Pieters MS, Kroon R, Breimer DD, Cohen AF. A comparison of the sensitivities of adaptive tracking, eye movement analysis and visual analog lines to the effects of incremental doses of temazepam in healthy volunteers. *Clin Pharmacol Ther.* 1991;50:172-180.

8 Van Steveninck AL, van Berckel BN, Schoemaker RC, Breimer DD, van Gerven JM, Cohen AF. The sensitivity of pharmacodynamic tests for the central nervous system effects of drugs on the effects of sleep deprivation. *J Psychopharmacol.* 1999;13:10-17.

9 Van Steveninck AL. Methods of assessment of central nervous system effects of drugs in man. State University Leiden; 1994.

10 Baloh RW, Sills AW, Kumley WE, Honrubia V. Quantitative measurement of saccade amplitude, duration, and velocity. *Neurology.* 1975;25:1065-1070.

11 Van Steveninck AL. A microcomputer based system for recording and analysis of smooth pursuit and saccadic eye movements. Cohen AF WT, editor. Brit.J.Clin.Pharmaco. 27[5], 712-713. 1989.

12 Norris H. The action of sedatives on brain stem oculomotor systems in man. *Neuropharmacology.* 1971;10:181-191.

13 Van Steveninck AL. Methods of assessment of central nervous system effects of drugs in man. Thesis, State University Leiden . 1993.

14 Tschirch FT, Gopfert K, Frohlich JM, Brunner G, Weishaupt D. Low-dose intranasal versus oral midazolam for routine body MRI of claustrophobic patients. *Eur Radiol.* 2007;17:1403-1410.

15 Shannon M, Albers G, Burkhart K, Liebelt E, Kelley M, McCubbin MM, et al. Safety and efficacy of flumazenil in the reversal of benzodiazepine-induced conscious sedation. The Flumazenil Pediatric Study Group. *J Pediatr.* 1997;131:582-586.

16 Overly FL, Wright RO, Connor FA, Jay GD, Linakis JG. Bispectral analysis during deep sedation of pediatric oral surgery patients. *J Oral Maxillofac Surg.* 2005;63:215-219.

17 Marx CM, Stein J, Tyler MK, Nieder ML, Shurin SB, Blumer JL. Ketamine-midazolam versus meperidine-midazolam for painful procedures in pediatric oncology patients. *J Clin Oncol.* 1997;15:94-102.

18 Louon A, Reddy VG. Nasal midazolam and ketamine for paediatric sedation during computerised tomography. *Acta Anaesthesiol Scand.* 1994;38:259-261.

19 Ljungman G, Kreuger A, Andreasson S, Gordh T, Sorensen S. Midazolam nasal spray reduces procedural anxiety in children. *Pediatrics.* 2000;105:73-78.

20 Liu J, Singh H, White PF. Electroencephalogram bispectral analysis predicts the depth of midazolam-induced sedation. *Anesthesiology.* 1996;84:64-69.

21 Lejus C, Renaudin M, Testa S, Malinovsky JM, Vigier T, Souron R. Midazolam for premedication in children: nasal vs. rectal administration. *Eur J Anaesthesiol.* 1997;14:244-249.

22 Hollenhorst J, Munte S, Friedrich L, Heine J, Leuwer M, Becker H, et al. Using intranasal midazolam spray to prevent claustrophobia induced by MR imaging. AJR *Am J Roentgenol.* 2001;176:865-868.

23 Connors K, Terndrup TE. Nasal versus oral midazolam for sedation of anxious children undergoing laceration repair. *Ann Emerg Med.* 1994;24:1074-1079.

24 Calderon E, Pernia A, Roman MD, Perez AC, Torres LM. [Analgesia and sedation in the subarachnoid anesthesia technique: comparative study between remifentanil and fentanyl/midazolam]. *Rev Esp Anestesiol Reanim.* 2003;50:121-125.

25 Bergese SD, Patrick BS, McSweeney TD, Fernandez S, Dzwonczyk R, Sage K. A comparative study of dexmedetomidine with midazolam and midazolam alone for sedation during elective awake fiberoptic intubation. *J Clin Anesth.* 2010;22:35-40.

26 Avramov MN, Smith I, White PF. Interactions between midazolam and remifentanil during monitored anesthesia care. *Anesthesiology.* 1996;85:1283-1289.

27 Wezenberg E, Sabbe BG, Hulstijn W, Ruigt GS, Verkes RJ. The role of sedation tests in identifying sedative drug effects in healthy volunteers and their power to dissociate sedative-related impairments from memory dysfunctions. *J Psychopharmacol.* 2007;21:579-587.

28 Motsch J, Epple J, Fresenius M, Neff S, Schmidt W, Martin E. [Desflurane versus isoflurane in geriatric patients. A comparison of psychomotor and postoperative well-being following abdominal surgical procedures]. *Anasthesiol Intensivmed Notfallmed Schmerzther.* 1998;33:313-320.

29 Polster MR, Gray PA, O'Sullivan G, McCarthy RA, Park GR. Comparison of the sedative and amnesic effects of midazolam and propofol. *Br J Anaesth.* 1993;70:612-616.

30 Dudchenko P, Paul B, Sarter M. Dissociation between the effects of benzodiazepine receptor agonists on behavioral vigilance and responsitivity. *Psychopharmacology (Berl).* 1992;109:203-211.

31 Chernik DA, Gillings D, Laine H, Hendler J, Silver JM, Davidson AB, et al. Validity and reliability of the Observer's Assessment of Alertness/Sedation Scale: study with intravenous midazolam. *J Clin Psychopharmacol.* 1990;10:244-251.

32 Deng XM, Xiao WJ, Luo MP, Tang GZ, Xu KL. The use of midazolam and small-dose ketamine for sedation and analgesia during local anesthesia. *Anesth Analg.* 2001;93:1174-1177.

33 Smith MT, Eadie MJ, Brophy TO. The pharmacokinetics of midazolam in man. *Eur J Clin Pharmacol.* 1981;19:271-278.

34 Allonen H, Ziegler G, Klotz U. Midazolam kinetics. *Clin Pharmacol Ther.* 1981;30:653-661.

35 Burstein AH, Modica R, Hatton M, Forrest A, Gengo FM. Pharmacokinetics and pharmacodynamics of midazolam after intranasal administration. *J Clin Pharmacol.* 1997;37:711-718.

36 Hardmeier M, Zimmermann R, Ruegg S, Pfluger M, Deuster S, Suter K, et al. Intranasal Midazolam: Pharmacokinetics and Pharmacodynamics Assessed by

Quantitative EEG in Healthy Volunteers. *Clin Pharmacol Ther.* 2012.
37 Haschke M, Suter K, Hofmann S, Witschi R, Frohlich J, Imanidis G, et al. Pharmacokinetics and pharmacodynamics of nasally delivered midazolam. *Br J Clin Pharmacol.* 2010;69:607-616.
38 Wermeling DP, Record KA, Kelly TH, Archer SM, Clinch T, Rudy AC. Pharmacokinetics and pharmacodynamics of a new intranasal midazolam formulation in healthy volunteers. *Anesth Analg.* 2006;103:344-9, table.
39 Knoester PD, Jonker DM, Van Der Hoeven RT, Vermeij TA, Edelbroek PM, Brekelmans GJ, et al. Pharmacokinetics and pharmacodynamics of midazolam administered as a concentrated intranasal spray. A study in healthy volunteers. *Br J Clin Pharmacol.* 2002;53:501-507.
40 Dale O, Nilsen T, Loftsson T, Hjorth TH, Klepstad P, Kaasa S, et al. Intranasal midazolam: a comparison of two delivery devices in human volunteers. *J Pharm Pharmacol.* 2006;58:1311-1318.
41 Gudmundsdottir H, Sigurjonsdottir JF, Masson M, Fjalldal O, Stefansson E, Loftsson T. Intranasal administration of midazolam in a cyclodextrin based formulation: bioavailability and clinical evaluation in humans. *Pharmazie.* 2001;56:963-966.
42 Loftsson T, Gudmundsdottir H, Sigurjonsdottir JF, Sigurdsson HH, Sigfusson SD, Masson M, et al. Cyclodextrin solubilization of benzodiazepines: formulation of midazolam nasal spray. *Int J Pharm.* 2001;212:29-40.
43 de Visser SJ, van der Post JP, de Waal PP, Cornet F, Cohen AF, van Gerven JM. Biomarkers for the effects of benzodiazepines in healthy volunteers. *Br J Clin Pharmacol.* 2003;55:39-50.
44 Aho M, Erkola O, Kallio A, Scheinin H, Korttila K. Comparison of dexmedetomidine and midazolam sedation and antagonism of dexmedetomidine with atipamezole. *J Clin Anesth.* 1993;5:194-203.
45 Ayuse T, Hoshino Y, Kurata S, Ayuse T, Schneider H, Kirkness JP, et al. The effect of gender on compensatory neuromuscular response to upper airway obstruction in normal subjects under midazolam general anesthesia. *Anesth Analg.* 2009;109:1209-1218.
46 Ball DM, Glue P, Wilson S, Nutt DJ. Pharmacology of saccadic eye movements in man. 1. Effects of the benzodiazepine receptor ligands midazolam and flumazenil. *Psychopharmacology (Berl).* 1991;105:361-367.
47 Bevan JC, Veall GR, Macnab AJ, Ries CR, Marsland C. Midazolam premedication delays recovery after propofol without modifying involuntary movements. *Anesth Analg.* 1997;85:50-54.
48 Salmon JF, Mets B, James MF, Murray AD. Intravenous sedation for ocular surgery under local anaesthesia. *Br J Ophthalmol.* 1992;76:598-601.
49 Song YS, Song ES, Lee KH, Park YH, Shin WC, Ku JH. Sleep-related nocturnal erections and erections during midazolam-induced sedation in healthy young men. *Int J Impot Res.* 2006;18:522-526.
50 Sundstrom I, Nyberg S, Backstrom T. Patients with premenstrual syndrome have reduced sensitivity to midazolam compared to control subjects. *Neuropsychopharmacology.* 1997;17:370-381.
51 van Gerven JM, Roncari G, Schoemaker RC, Massarella J, Keesmaat P, Kooyman H, et al. Integrated pharmacokinetics and pharmacodynamics of Ro 48-8684, a new benzodiazepine, in comparison with midazolam during first administration to healthy male subjects. *Br J Clin Pharmacol.* 1997;44:487-493.
52 Griffiths AN, Marshall RW, Richens A. Saccadic eye movement analysis as a measure of drug effects on human psychomotor performance. *Br J Clin Pharmacol.* 1984;18 Suppl 1:73S-82S.

TABLE 1 Pharmacokinetic parameters of midazolam and α-hydroxy-midazolam in healthy subjects after administration of a single dose of 2.5 mg midazolam intravenous (i.v.) or 2.5 or 5 mg midazolam intranasal (i.n.)

	Treatment	AUC$_{0-\infty}$ (ng*h ml^{-1})*	C_{max} (ng ml^{-1})	$t_{1/2}$ (h)*	T_{max} (min)	F*
MIDAZOLAM	Midazolam 2.5 mg i.v.	93.9 (33.8)	219.2 (68.1)	3.6 (29.4)	2.0 (1.2-3.0)	1
	Midazolam 2.5 mg i.n.	65.6 (49.0)	30.6 (42.3)	6.3 (123.4)	10.9 (6.0-24.0)	0.74 (0.28-1.85)
	Midazolam 5 mg i.n.	131.9 (26.0)	66.2 (31.5)	4.3 (31.0)	13.8 (9.0-24.0)	0.76 (0.45-1.20)
A-HYDROXY-MIDAZOLAM	Midazolam 2.5 mg i.v.	15.83 (36.9)	6.1 (37.2)	4.6 (45.5)	14.4 (9.0-21.0)	
	Midazolam 2.5 mg i.n.	10.9 (54.1)	2.4 (55.5)	5.3 (40.0)	45.4 (24.0-240.0)	
	Midazolam 5 mg i.n.	24.0 (37.5)	5.3 (34.5)	6.3 (44.2)	50.6 (21.0-121.2)	

AUC, C_{max} and half-lives are expressed as geometric mean (CV%); T_{max} and F are expressed as geometric mean (range); AUC, area under the curve; Cmax, peak plasma concentration; CV, coefficient of variation, F, bioavailability, i.v., intravenous; i.n. intranasal; $t^{1/2}$, elimination half-life; T_{max}, time to reach Cm.

FIGURE 1 Geometric mean concentration-time profiles (log-linear) of midazolam and α-hydroxy-midazolam after intravenous (IV) (2.5 mg) and intranasal (IN) (2.5 and 5.0 mg) midazolam administration in healthy subjects

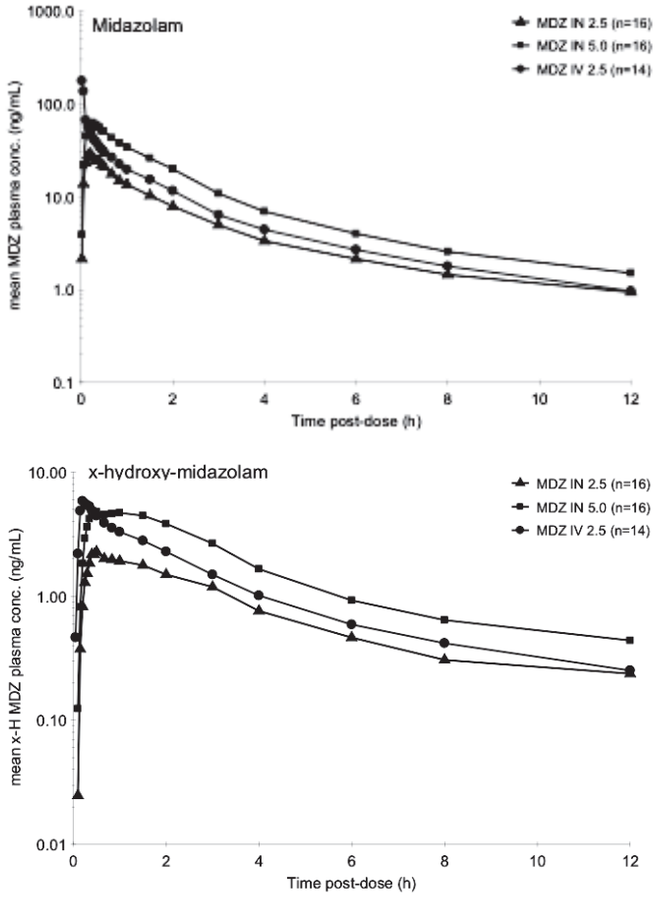


FIGURE 2 Saccadic Peak Velocity LSMs change from baseline profile with 95% CI as error bars (first 3 hours after administration). Open rhombus represents placebo; grey closed circle represents midazolam 2.5 mg i.v.; black closed circle represents midazolam 2.5 mg i.n.; open circle represents midazolam 5.0 mg i.n.

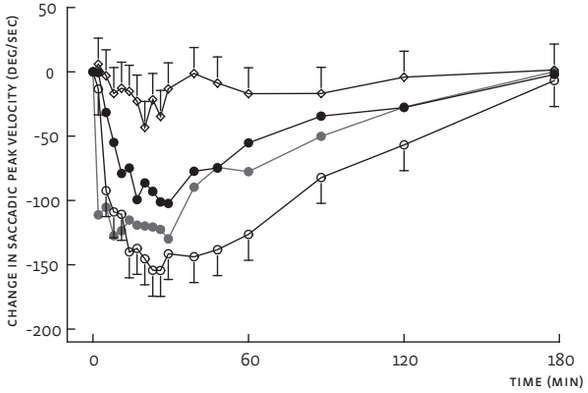


FIGURE 3 VAS Alertness LSMs change from baseline profile with 95% CI as error bars (first 3 hours after administration). Open rhombus represents placebo; grey closed circle represents midazolam 2.5 mg i.v.; black closed circle represents midazolam 2.5 mg i.n.; open circle represents midazolam 5.0 mg i.n.

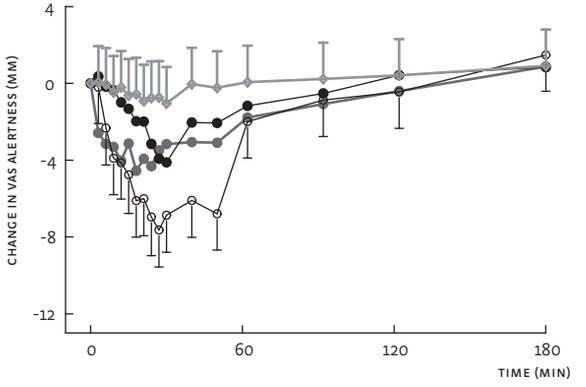


FIGURE 4 Simple reactions time task LSMs change from baseline profile with 95% CI as error bars (first 3 hours after administration). Open rhombus represents placebo; grey closed circle represents midazolam 2.5 mg i.v.; black closed circle represents midazolam 2.5 mg i.n.; open circle represents midazolam 5.0 mg i.n.

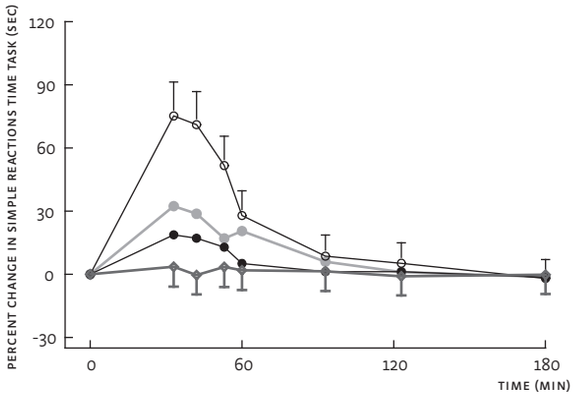
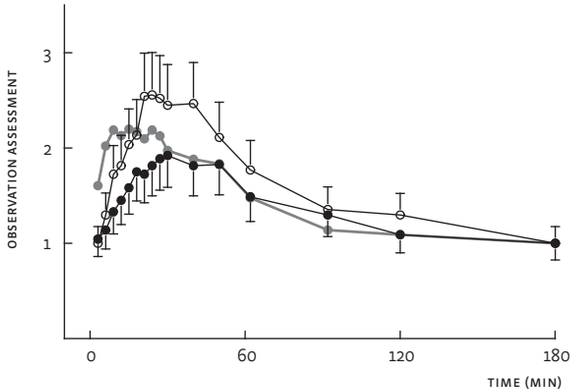


FIGURE 5 Observation assessment LSMs profile with 95% CI as error bars (first 3 hours after administration). Grey closed circle represents midazolam 2.5 mg i.v.; black closed circle represents midazolam 2.5 mg i.n.; open circle represents midazolam 5.0 mg i.n. Score 1 = awake/oriented; score 2 = drowsy/normal speech; score 3 = slow reaction to verbal; score 4 = inability 2 saccades



CHAPTER 10

General discussion

CNS drug research under the Pediatric Regulation: how can we move forward?

The 2007 EU Pediatric Regulation requires the industry to plan clinical trials in children early in the development of new drugs for use in adults, or for line extensions for on-patent drugs (unless a waiver or deferral has been granted). Because the Regulation is at the core of pediatric needs and establishes clear obligations and a system of incentives aimed at the pharmaceutical industry, the Regulation was expected to result in research that would be focused more on the needs of children and would therefore drive important changes in therapeutic options available to pediatric patients[1]. Based on our evaluation presented in chapter 2, it is clear that under the Regulation, a higher percentage of medicines are considered for use in children. However, the Regulation does not necessarily lead to the increased pediatric development of drug classes for which there may be a unmet pediatric need based on pediatric usage and availability data. Importantly, only seven therapeutic subgroups accounted for half of all medicines for which pediatric development was agreed, and the drug classes that the European Medicines Agency (EMA) Needs Lists identified as needing pediatric research and development were researched relatively infrequently under the Regulation. In addition, a small contingent of Dutch physicians working in pediatric healthcare are not convinced that medicines for which pediatric development was agreed are needed for clinical practice.

Our analysis confirms the increased need for researching and developing central nervous system (CNS) drugs for use in pediatric patients, as drugs acting on the nervous system (the so-called neurologicals) had the highest number of off-label medicines for which the EMA Needs Lists identified a need[2]. Nearly half of all CNS drugs listed were indicated to need full pediatric development. Anesthetics, analgesics, antiepileptics, and psycholeptics (such as antipsychotics, anxiolytics, hypnotics, and sedatives) were among the therapeutic subgroups with the highest number of active substances with a pediatric need. Our analysis also indicates that the percentage and number of children and adolescents who are treated using CNS drugs continues to rise, reflected by an annual increase in out-patient use of neurologicals in Dutch children and adolescents from 2005 through 2011 (the end of the study period). The largest increase was among antidepressants (including antidepressants combined with psycholeptics) and stimulants. The in-patient use of CNS drugs was not evaluated, but is also likely substantial, given that neonatologists, pediatricians, pediatric neurologists, and child and adolescent psychiatrists commonly prescribe neuropsychiatric drugs.

Despite this high need for pediatric research and development, and despite the ongoing increase in the use of CNS drugs among pediatric patients, CNS drugs are researched only rarely under the Regulation. In addition, the Regulation is not likely to have a positive impact on the ability of children and adolescents to access new CNS drugs, or on the delay in pediatric registration of CNS drugs as described in the Introduction to this thesis. A total of 32 CNS drugs were agreed for pediatric development, the majority of which (approximately 80%) had a unique Anatomical Therapeutic Chemical (ATC) code in the WHOCC (World Health Organization Collaborating Center) database (not reported in chapter 2), indicating that these drugs are currently marketed in at least one country. The newly developed CNS drugs included several novel drugs; however, the development of some of these drugs had already been discontinued[3]. In addition, for the majority (81%) of CNS drugs with agreed pediatric development, one or more pediatric development plans included at least one measure for which deferral was granted until the drug was authorized for marketing to adults. The relatively low number of CNS drugs with agreed pediatric development is likely due to the reported decrease in research in adults. Because the adult indication is the starting point of the Regulation, and because pediatric development plans are submitted as part of a development plan in adults, drug development for children with neuropsychiatric disorders still follows adult development, although some pediatric epilepsy syndromes (such as neonatal seizures, Lennox-Gastaut syndrome and Dravet syndrome) are being addressed specifically under the Regulation.

To address some of these issues, in chapter 2 we suggested that the evaluation of any proposal for a pediatric investigation plan (or request for a waiver)

should be based on the potential pediatric relevance of the mechanism of action or drug target, and new incentives should be considered for first-in-children indications. In addition, given the high number of CNS drugs with deferred pediatric studies, clinical trial strategies should be revised in a timely fashion. Deferrals under the Pediatric Regulation may be related in part to the need for extra time due to a lack of general expertise, as pediatric research in the EU is less extensive than in the United States. For example, in the field of child and adolescent psychopharmacology, the majority of publications and studies originate in the US[4], and the ability to run early drug trials with innovative therapies is significantly higher in the US than in Europe[5]. Because clinical research in pediatric patients is hampered by interrelated logistic and ethical constraints – including a limited number, extent, and invasiveness of study-related interventions that can be performed if they are not part of routine clinical care – researchers should attempt to reduce the burden placed on participating children and adolescents by using non-invasive or minimally invasive measurement methods. Changes in methods that are designed to reduce the patient's burden (for example, changing the sampling procedure) have already been reported to increase patient enrollment in studies of rare pediatric diseases that were performed under the Regulation[6]. Therefore, in this thesis, we explored the feasibility of using non-invasive monitoring of pharmacokinetics (PK) and pharmacodynamics (PD) for pharmacological drug profiling of commonly used CNS stimulants and depressants in children and/or adolescents. Drug concentrations were measured non-invasively in either the saliva or exhaled breath, and neurocognitive and neurophysiological functions were measured longitudinally using the NeuroCart test battery.

This chapter reviews how this approach was used in the preceding chapters of this thesis. We will first discuss the feasibility and applicability of saliva sampling in pediatric populations based on data obtained from pediatric clinical studies of caffeine, methylphenidate, and melatonin. Then, we will discuss the (potential of) pharmacological profiling of pharmacodynamics in children and adolescents based on literature review and clinical studies of methylphenidate, caffeine, alcohol and midazolam. This chapter concludes with potential practical applications of this approach and suggestions for future directions.

Non-invasive drug profiling in children and adolescents

PHARMACOKINETICS

In chapters 6 and 8, we evaluated the feasibility and applicability of sampling saliva from healthy adolescents and from children and adolescents with autism spectrum disorder (ASD). Although the sample collection devices and techniques (for example active versus passive collection) are dictated by the characteristics of the drug of interest, the collection method must be tolerated by children and adolescents. As we expected, active sampling using a Salivette collection device was tolerated well by the adolescents who participated in the caffeine study (chapter 6). Children with ASD can have particularly sensitive sensory systems[7] that can cause the child to resist certain collection devices. In the study described in chapter 8, we collected whole saliva samples from children and adolescents with ASD; whole saliva sampling is the preferred method for measuring melatonin concentrations in saliva[8]. A recent study of 6-12-year-old (mostly male) children with ASD found that passive saliva sampling was an acceptable collection method (in terms of ease and comfort)[9], and this method can also be used in young children[10] without associated risks such as choking. In our study, we used the Saliva Collection Aid (available from Salimetrics Europe), a device that was recently developed to simplify the collection of whole saliva from passive drool. Importantly, the device is constructed of polypropylene, which resists sample retention and contamination, issues that can occur with amines such as melatonin. This collection method was tolerated well by the subjects in our study.

Due to difficulties in reliably predicting plasma concentration using saliva measurements, determining the concentration of a drug in a saliva sample was limited for basic drugs (which undergo an alkaline reaction in aqueous solutions) and for acidic drugs and drugs that are highly protein-bound (these drugs can have an extremely low S/P ratio). However, if sources of variability in the S/P ratio can be overcome for these drug types, measuring saliva drug concentration might be a feasible alternative to measuring plasma drug concentration. The studies described in chapters 5 and 6 attempted to investigate and quantify the sources of variability (for example, contamination and

saliva pH) in the plasma and saliva MPH and caffeine concentrations measured using a population PK modeling approach. In previous studies, the S/P ratios of MPH[11] and caffeine[12–14] concentrations were time-dependent, possibly due to fluctuations in arteriovenous blood concentration[12–14] and/or pH partitioning[11,13]. In addition, because many drugs are administered orally, contamination in the saliva can influence the S/P ratio at early time points after administration. In chapters 5 and 6, we showed that the relationships between plasma and saliva concentrations of both MPH and caffeine are stable (i.e., not time-dependent or concentration-dependent) after 2.5 hours (for MPH) and one hour (for caffeine) of administration. Prior to these time points, the relationship between plasma and saliva concentrations was not linear.

Sample contamination due to residual drug levels is a general concern in pediatric clinical research, but this issue is rarely addressed or taken into account. For example, in oncology research, PK measurements are usually taken from samples obtained directly from the indwelling central venous line (CVL), which is the same line through which intravenous chemotherapeutics are administered. This is usually due to practical considerations (as the line is already in place) and prevents the need to collect samples from a peripheral catheter (which would need to be inserted), thereby increasing study enrollment. Although studies suggest that clearing the line can minimize the contamination of certain drugs, there is currently no universally accepted method to reliably address this issue. One exception is a recently published paper by Edwards and colleagues[15], who developed a population PK model of actinomycin-D in children with cancer by incorporating expressions that account for drug contamination from samples obtained via an indwelling CVL. Compared to other models, their baseline contamination model – including a contamination factor proportional to the model-predicted concentration for samples obtained from a CVL – was chosen as the most conservative and accurate model. This contamination model assumed that drug contamination from the sampling catheter was included in the baseline concentration and was therefore factored into the level of each individual prediction. Because many CNS drugs are delivered orally, sample contamination can also play a role when using saliva sampling. For example, remnants of syrup, uncoated tablets, or chewable tablets can contaminate saliva samples and can cause inappropriately high drug concentrations in samples measured early after administration. This source of contamination can be largely eliminated by thoroughly rinsing the mouth both after taking the drug and prior to obtaining a saliva sample[16], as was done in the studies described in this thesis. However, even if the mouth is rinsed thoroughly, saliva concentrations can be higher than expected due to residual contamination, transient transmucosal absorption or deposition of the drug. Therefore, in the studies described in chapters 5 and 6, we attempted to compare the magnitude of contamination after drinking a beverage (caffeine) or swallowing a tablet (an immediate-release MPH tablet) with contamination after swallowing a sealed capsule. Using this approach, we anticipated that a contamination factor (the elimination rate constant) could be incorporated into the model equation describing the saliva PK data, and model-predicted saliva concentrations (and hence the S/P ratio) could be individually corrected for the level of contamination. In addition, the half-life of the elimination rate constant could be used to determine the time window in which considerable contamination could be expected, thus providing information regarding sampling time points for therapeutic drug monitoring in clinical practice. Unfortunately, our efforts to correct for oral contamination were largely unsuccessful, due in part to the relatively few observations measured at early time points following drug administration; therefore, this approach was abandoned.

Even in cases in which the saliva sample is not contaminated, the S/P ratio may not be consistent across PK phases. Several factors may account for this inconsistency[17]. First, the arteriovenous concentration ratio can vary between PK phases, particularly for compounds that diffuse easily. Second, even in the absence of this phenomenon, the S/P ratio may not be stable throughout all PK phases. If elimination is linear and proceeds at the same rate in plasma and saliva (i.e., parallel decline), the ratio will be inconsistent; however, if elimination proceeds exponentially, the ratio will remain constant, with a parallel decline in plasma and saliva drug concentrations. Third, concentration-dependent

protein binding can account for differences in the S/P ratio within an individual subject. Finally, pH partitioning can play a role, particularly for basic drugs, which undergo an alkaline reaction in aqueous solutions. Many CNS drugs are basic and reside in equilibrium between their charged and neutral states under physiological conditions. Thus, the free fraction of the ionized drug can be incorporated in saliva, as saliva is slightly more acidic than plasma[18]. Therefore, the S/P ratio is highly sensitive to small changes in saliva pH, which in turn can be influenced by saliva flow. In the studies described in chapters 5 and 6, we measured saliva pH and saliva flow at each saliva sampling time point in order to determine whether changes in these parameters (despite active sampling) accounted for some of the residual variability in the S/P ratio. However, no significant covariates were identified for the S/P ratio.

The feasibility of using non-invasive saliva samples to measure PK is limited by the period of time needed by the subject between sampling time points to replace the saliva sampled. Thus, the number of samples that can be collected to measure the absorption phase of compounds with a short T_{max} (such as MPH and caffeine) is limited, thus hampering the ability to determine the magnitude of contamination, the S/P ratio, and sources of variability in the S/P ratio directly following administration. For these drug types, only CNS effects that occur relatively late are suitable for developing a PK/PD model using measured saliva concentrations or predicted plasma concentrations. However, because the pharmacological CNS action of most compounds is delayed relative to changes in plasma concentration, saliva sampling may still be a viable option for this type of research. Therefore, our results support the further exploration of using saliva as a non-invasive, feasible, and acceptable method for drug profiling CNS drugs. Given the potential influence of saliva contamination and saliva pH on measured saliva drug concentration, standardizing the collection method (for example, by rinsing the mouth, choosing active versus passive sampling, etc.) will likely improve the applicability of using saliva for drug monitoring. On the other hand, the method is potentially limited by the fact that the relatively low volume of saliva in a sample necessitates the use of a highly sensitive bioanalytical method such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), which can be costly and is currently not generally available for most CNS drugs.

PHARMACODYNAMICS

The most accessible non-invasive method for assessing the effect of a CNS drug is to measure drug-related CNS functional activity with sufficient sensitivity and specificity[19]. In general, an excessive number of CNS tests are currently used in psychopharmacological research to determine the effects of drugs that act on the nervous system. The sensitivity of these tests for assessing CNS effects has not been determined fully for most drugs, and the reproducibility of these tests may be relatively low. Therefore, careful selection of PD parameters is essential. In the studies described in this thesis, useful CNS tests (or functional biomarkers) to measure the effects of several compounds (including alcohol[20], benzodiazepines[21], and others) in healthy subjects were selected based on previously published studies. These parameters were used to measure PD for alcohol and midazolam in studies of healthy volunteers; these studies are described in chapters 7 and 9, respectively.

Selecting functional biomarkers for measuring drug effects in patients with neuropsychiatric disorders is likely to be more complicated than in healthy volunteers. A useful functional biomarker should be sensitive enough to detect a therapeutic drug dose; in addition, a plausible relationship between the biomarker, drug pharmacology, and/or disease pathophysiology should also be evident[22-26]. Because the precise mechanism of action of many CNS drugs is not fully understood, and because many neuropsychiatric disorders are heterogeneous, these two criteria may be difficult to achieve. The review reported in chapter 3 describes a systematic literature search that used the same approach as previously published reviews in healthy subjects. The aim of our review was to assess the sensitivity and usefulness of functional biomarkers for demonstrating acute CNS effects of immediate-release methylphenidate (MPH-IR) in children and adolescents with attention-deficit/hyperactivity disorder (ADHD). Pediatric ADHD is an exception in the field of pediatric neuropsychopharmacology, as an extensive body of research has been performed

to study the effects of MPH-IR (and other drugs) in this disorder. However, previously published studies investigating the effects of MPH-IR yielded contradictory results due to several sources of variability, including a lack of standardized biomarkers and/or effect measures of MPH[27,28]. The results of our review revealed that a wide variety of biomarkers are currently being used to evaluate the effect of MPH-IR in ADHD. In addition, our review revealed that these studies would benefit greatly from a certain degree of standardization. Because most tests were used relatively rarely, it was difficult to identify the most sensitive tests and drug–response relationships. Nevertheless, despite these limitations, our literature review revealed that the Go/no-go task, the Scale-ADHD, and tests that assess motor control and/or sustained attention may be suitable candidate biomarkers to measure the acute effects of MPH-IR. These tests may facilitate the identification of responders and non-responders following a test dose of MPH-IR. Our evaluation shows that even in the context of extensive pediatric research, it is difficult to reach useful conclusions regarding pharmacological profiles or dose-effect relationships based on currently available studies. For example, several studies in our analysis did not measure drug concentrations, or they performed only a single PD measurement following the test dose. Future studies are needed in order to investigate effect size and to establish clinically relevant changes in CNS test results. Ideally, these studies should include concentration-effect and dose-effect relationships measured at several time points in order to allow for drug effect profiling, similar to the approach used in the clinical studies described in this thesis.

The CNS tests that are included in the NeuroCart battery are sensitive enough to detect low doses of caffeine and alcohol in adolescents, as shown by the studies described in chapters 6 and 7. In the study described in chapter 6, an extensive CNS battery was incorporated in order to obtain information regarding general CNS performance and to identify the functional CNS domains that are affected by caffeine. The CNS tests included saccadic and smooth pursuit eye movements, body sway, adaptive tracking, the left/right distraction task, finger tapping, the attention switch task and the visual and verbal learning task. In addition, blood pressure and heart rate were measured at regular intervals after the administration of caffeine. Significant effects on parameters regarding alertness (saccadic peak velocity) and reaction time (adaptive tracking) were observed after caffeine was administered, despite the relatively low dose and the expected ceiling effect on several parameters in this cohort of healthy, alert adolescents. Improved postural stability and some of the response times approached the level of statistical significance; thus, it is likely that these functions will improve significantly at higher caffeine doses. Because caffeine has been reported to produce behavioral effects (including motor activation and arousal) similar to the effect of classic psychostimulants such as cocaine and amphetamine[29], these tasks could be valuable in pediatric studies using other CNS stimulants. In the study described in chapter 7, a low dose of alcohol induced significant changes in smooth pursuit eye movements, alertness score measured on the visual analog scale (VAS), the VAS alcohol effect score, body sway, systolic blood pressure, and heart rate. Given that animal studies have revealed developmental changes in the pharmacological sensitivity of $GABA_A$-receptor-mediated currents to several drugs (including diazepam[30,31], pentobarbital[30], and zolpidem[32–35]), these tasks could be used to study age-related differences in the CNS effects of GABA-ergic compounds in pediatric drug research. The CNS tests and their duration were tolerated by the majority of adolescent subjects in these clinical studies. These studies demonstrate the feasibility of collecting a rich dataset from this age group, and they show that neuropsychological and psychomotor tasks can be used as biomarkers of the acute effects of low-dose caffeine or alcohol in healthy adolescents.

Potential practical applications

In the introduction (chapter 1) of this thesis, we raised several issues with respect to pediatric neuropsychopharmacology. First, the differences in both neuropsychopathology and pharmacology between children and adults must be recognized. In addition, researchers need validated tools that are

appropriate for assessing the efficacy and safety of CNS drugs in children. Finally, special emphasis should be placed on formulation research. CNS drug profiling has the potential to address all of these issues, as it enables researchers to evaluate age-dependent differences in PK and PD, and it can facilitate distinguishing between the drug properties of sedatives and stimulants in children. In addition, CNS drug profiling can facilitate comparisons between profiles of drugs with different formulations.

Because our current understanding of age-dependent effects and side-effects is based largely on data collected from animal studies, evaluating age-dependent differences in PK and PD in pediatric clinical trials will be an important step forward. Drug profiling enables researchers to evaluate age-dependent effects by drawing comparisons with existing data collected in adults, as demonstrated by the study described in chapter 7. In this study, we used a PK/PD modeling approach to compare the alcohol breath profiles of adolescents and adults, as well as the participants' objective and subjective responses to alcohol. Oral data collected from adolescents were combined in a PK/PD model with previous intravenous data collected from adults. Inter-subject variability could be identified on the basis of various kinetic parameters, and all of the identified covariates were related—either directly or indirectly—to differences between adolescents and adults. Two functional biomarkers were selected based on an exploratory meta-analysis of all PD tasks that were performed several times during a single testing occasion (from several studies). Both smooth pursuit eye performance and VAS alertness responded clearly to alcohol, with no indication of any indirect effects or acute tolerance; thus, it was likely that a relatively simple model would describe the data accurately, and the presence or absence of an age-dependent effect could be investigated. Although we successfully developed a PK/PD model for these functional biomarkers, we did not identify any significant covariates; in particular, we found no clear effect of age. However, VAS alertness and smooth pursuit eye movements may not fully represent every effect of alcohol on the CNS, and we cannot exclude the possibility that sensitivity to other pharmacodynamic effects of alcohol changes with age. Because age-dependent differences may exist for functional biomarkers of acute tolerance (such as subjective intoxication) and/or for tests that evaluate postural stability, it is worth investigating whether sensitivity to other pharmacodynamic effects of alcohol change with age.

Several drug classes—including adenosine antagonists; noradrenergic and serotonergic reuptake inhibitors; $GABA_A$-receptor agonists; ethanol; cannabinoid agonists and antagonists; dopamine antagonists; histamine antagonists; and muscarinic antagonists—have a unique CNS 'fingerprint' (i.e. drug profile) on the NeuroCart test battery. This unique drug profile corresponds to the drug class' mechanism of action[36]. These "fingerprints' can be used to differentiate a drug's stimulant and sedative properties. For example, CNS stimulants can exert a different—or even opposite—effect profile than CNS depressants. CNS stimulants temporarily increase mental and/or physical function and are believed to act primarily on the dopamine, noradrenaline, and serotonin systems or by disinhibiting adenosine. Thus, CNS stimulants may have a common pharmacological profile. By analyzing the results of studies performed previously by our research group and by reviewing other studies (de Mol, unpublished data), we found previously that increased saccadic peak velocity is a typical sign of CNS stimulation. In addition, increased adaptive tracking performance and elevated body temperature were also identified as likely signs of CNS stimulation. Given that benzodiazepines—which are typical CNS sedatives—and ethanol are reported to decrease saccadic peak velocity[23,36], decrease adaptive tracking performance[23], and induce hypothermia[22,37–39], these functional biomarkers might be valuable for providing a better interpretation of the CNS stimulant or sedative effects of drugs that have a modified or novel mechanism of action. In addition, these biomarkers can be used to quantify and compare these properties (including paradoxical side-effects such as agitation in the case of benzodiazepines) between children and adults. For example, functional biomarkers could be used to determine whether a specific drug exerts a pharmacological effect at a specific dose in children, and they could also be used to monitor unwanted sedative or stimulant properties of drugs in children.

Finally, non-invasive monitoring of PK and PD could facilitate the evaluation of age-appropriate formulations of CNS stimulants and depressants for use in children, as illustrated by the studies described in chapters 8 and 9. Because oral delivery remains the most important and most commonly used route for drug delivery, suitable oral delivery forms that offer the possibility of customized dosing are urgently needed[40]. Sustained-release mini-tablets provide several distinct advantages over other conventional pediatric dosage forms; for example, mini-tablets can minimize dysphagia-related issues, they can be designed to mask unpleasant taste (thereby improving palatability and patient compliance), and they can be modified to release the active ingredient slowly[41]. In addition, mini-tablets can provide multiparticulate dosing, which is needed for global pediatric drug therapy[42]. In a recent study, mini-tablets were the best accepted oral formulation in infants and preschool children and were significantly more often fully swallowed than the other oral formulations[43]. Therefore, the new, age-appropriate prolonged-release melatonin mini-tablets (Circadin) described in chapter 8 may provide important advantages over the currently licensed 2-mg tablets. In this study, we evaluated the PK profile, short-term safety, and acceptability of this formulation in 16 children and adolescents with both autism spectrum disorder (ASD) and a sleep disorder. In order to avoid interrupting the subject's sleep due to repeated nighttime saliva sampling, the melatonin was administered in the morning (rather than in the evening); this also minimized study-related burden. Melatonin concentrations were measured non-invasively in saliva samples, and the level of sedation was evaluated using the Observer's Assessment of Alertness/Sedation (OAA/S) scale. This validated scale was originally developed to more objectively measure the level of alertness in sedated subjects, and it has been shown to be both reliable and sensitive to the effects of drugs such as midazolam[44]. In addition, the OAA/S scale is comparable to the Ramsey scale, which is commonly used in pediatric intensive care units to assess the patient's level of consciousness[45]. The concentration of melatonin in the saliva peaked within two hours of receiving either a dose of 2-mg or 10-mg Circadin, and the melatonin level remained elevated for several hours thereafter. Circadin exposure in saliva was dose-linear and clearance in saliva was comparable between dose groups. Sedation after the 2-mg Circadin dose peaked at 2 hours, which is around the T_{max} time of the PK profile, demonstrating PK/PD correlation.

Non-invasive monitoring of an array of sensitive functional biomarkers can also be used to compare the effect profiles of different formulations, as was demonstrated in chapter 9. The 'proof-of-pharmacology' strategy described in this chapter may perhaps also be followed in pediatric studies on drugs for which extensive data exist on efficacy and safety, but for which there is only a need for an age-appropriate formulation. In the study described in chapter 9, the pharmacokinetics of two doses of a novel highly concentrated aqueous intranasal midazolam formulation (Nazolam) was characterized in healthy adult volunteers and related to several outcome variables for sedation. In addition, the onset and duration of the pharmacological effect were evaluated and compared to intravenous midazolam using SPV, as a relationship between SPV reduction and clinical efficacy has been described[21]. Effects were seen on PD outcome variables of sedation and clinically relevant levels of sedation as measured using OAA/S were achieved within minutes after administration. In addition, single administration was well tolerated and safe. This new formulation has potential for use in conscious sedation and epilepsy in children. A future study in children could include non-invasive measurement of midazolam in saliva[46] and repeated effect measurements on SPV (in older children) and a clinically relevant biomarker of sedation (OAA/S). In addition, a previously validated anxiety score (Faces Pain Scale-revised)[47] could be used to measure the effect on anxiolysis.

FUTURE DIRECTIONS

In parallel with further refining the methods described in this thesis, several other steps must be taken. First, non-invasive drug monitoring should be evaluated in younger age groups and in subjects with other neuropsychiatric disorders. The studies described in this thesis primarily involved adolescents,

as the development of non-invasive drug monitoring generally follows an incremental 'top-down' approach, with adolescents studied first, followed by younger age groups. Adolescents are often overlooked in clinical trials that lead to drug registration[48]. Changes in physical parameters such as height, weight, and lean body mass occur during puberty, and these changes can strongly influence hepatic drug elimination and clearance during adolescence. Age-dependent changes in liver size and/or functional capacity can also contribute significantly to changes in clearance[49]. In addition, many adolescents are more amendable to certain medications due to neurochemical differences, and the presence or absence of significant side-effects can strongly influence whether the subjects comply with the drug administration regimen[50]. Recent studies found that adolescence is a unique developmental period of transition in which specific brain regions undergo highly dynamic growth and pruning[51]. Changing the levels of catecholamine neurotransmitters can have the strongest effect during the transition from childhood to adolescence, when synaptic selection peaks[52]. Because adolescents are rarely waived for pediatric development under the Pediatric Regulation, this age group will likely be involved in more studies in the near future. However, preschoolers have historically been the most neglected age group with respect to psychopharmacological research[53], and this age group is also likely to be included in more trials performed under the Regulation. Currently, the youngest age at which a child is treated by a child psychiatrist is 2-3 years (e.g., in the case of a young child with irritability or aggressive behavior, for example in ASD). Children with disorders such as pain or epilepsy may also require drug therapy at a younger age. Importantly, younger children may be more sensitive than older children to the adverse effects of certain medications. Pre-school children with ADHD may have lower effect sizes with MPH, and they may have a higher prevalence of adverse events[54], including increased insomnia or other sleep-related problems, reduced appetite[55], increased emotional levels, social withdrawal, nausea, and stomach ache[56]. Therefore, age-appropriate non-verbal CNS tests that do not require a long attention span should be developed and validated in order to evaluate CNS effects in preschool children. In addition, relying upon the subject to spontaneously report adverse events is often not appropriate in this age group, and third-party reporting of adverse events is often needed[57]. Therefore, more proactive, objective measures must be taken, for example by incorporating the OAA/S scale as reported in chapter 8.

A second important step, particularly considering that most neurological and psychiatric disorders are either chronic or recurrent, is to determine whether early treatment translates into an acute improvement in symptom-related CNS functions as well as improved function and improved long-term outcome. To achieve this goal, the predictive value of putative functional CNS biomarkers should be investigated in long-term trials. Given that many neurological and psychiatric disorders are highly heterogenous, a combination of tests should be included in such a trial, as combined testing can yield a more precise and robust prediction of the drug's effect. These studies should also include tolerability biomarkers, given that adverse events can reduce compliance or even cause the subject to discontinue treatment.

Finally, as anesthetics and analgesics are among the most commonly cited therapeutic subgroups with a pediatric research need on the EMA Needs Lists[2] (as reported in chapter 2), more experience should be gained in the field of pediatric pain research, especially as children are particularly prone to unfair exclusion from pain research[58]. Pupillometry may be a useful non-invasive method to objectively quantitate pain response/intensity in children[59]. However, there are some important questions in pain and analgesia research that cannot be answered without an evoked pain stimulus, and therefore the use of evoked pain modalities should also be expanded for use in pediatric clinical trials. Evoked pain offers important control over the environment and provides standardization of pain stimuli, thereby enabling a more rigorous exploration of individual differences or the environmental factors that can affect the subjective pain experience. In general, it is ethically preferable to avoid causing pain for research purposes, especially in minors. In addition, because the induction of pain can lack direct benefit, it is usually not deemed appropriate for use in either therapeutic pediatrich research or non-therapeutic pediatric research. However, as the factors that can affect the

relationship between drug concentration and pain relief can differ between children and adults, findings obtained from adult studies may not necessarily translate directly to children or even adolescents. For example, a study of school-age children who underwent a tonsillectomy found that paracetamol can have a robust placebo effect in this age group[60] and a child's thoughts and attitudes regarding pain can change with age, thereby contributing to more intense feelings of pain during adolescence than in childhood[61]. In addition, developmental changes in endogenous analgesic mechanisms and developmental modulation have been proposed in animals[62]. Reaction time and late laser-evoked brain potentials decrease from childhood to adulthood, which may reflect aspects of maturation in sensory processing by the thermoalgesic system[63]. Because our current understanding of age-dependent effects and side effects is based largely on data collected from animal studies, evaluating age-dependent differences in PK and PD in pediatric clinical trials will be an important step forward. Therefore, it is necessary to include adolescents and children in pain studies. We predict that evoked pain will be used increasingly in children and adolescents. Therefore, additional knowledge and experience should be gained in the pediatric field of evoked pain modalities, again following an incremental 'top-down' approach, with adolescents studied first, followed by younger age groups.

Conclusions

The clinical studies included in this thesis show that non-invasive drug profiling for CNS stimulants and depressants is feasible in healthy adolescents and children and adolescents with ASD. In addition, non-invasive drug profiling may help researchers both evaluate age-dependent PK and PD and compare the effect profiles of various formulations. Importantly, this approach may also facilitate the execution of studies included in Pediatric Investigation Plans under the Pediatric Regulation, thus representing an important step forward, particularly given the high need for pediatric research with respect to CNS drugs. The ideal pediatric study should be executable in the subjects' homes. Adult studies of novel drugs that could potentially be measured in saliva samples should include measurements of the S/P concentration ratio. Because considerable PK variability has been reported for several neuropsychiatric drugs, sensitive non-invasive or minimally invasive PD measurements should be assessed longitudinally. In addition, pediatric studies should ideally include a means to compare the results with adult studies. Finally, to expand our knowledge regarding the acceptability and tolerability of measurement methods, whenever possible the study participants should be asked to complete a questionnaire in order to collect information regarding the reasons why children and adolescents participate, as well as their perceived burden associated with the study.

REFERENCES

1 Pandolfini C, Bonati M. European paediatric research and children's therapeutic needs. A trial review. *Acta Paediatr.* 2008;97:1232-1237.
2 European Medicines Agency (PDCO). Pediatric Needs Lists. 2013.
3 Integrity Database. 2014.
4 Auby P. Pharmaceutical research in paediatric populations and the new EU Paediatric Legislation: an industry perspective. *Child Adolesc Psychiatry Ment Health.* 2008;2:38.
5 Vassal G. Will children with cancer benefit from the new European Paediatric Medicines Regulation? *Eur J Cancer.* 2009;45:1535-1546.
6 Winzenburg G. More than 5 years of European Paediatric Regulation: Statistics and industrial experience. *Int J Pharm.* 2014.
7 DSM-IV-TR. American Psychiatric Association (2000) Diagnostic and statistical manual of mental disorders 4th edition (DSM-IV-TR). American Psychiatric Association, Washington, DC; 2000.
8 Eriksson K, Ostin A, Levin JO. Quantification of melatonin in human saliva by liquid chromatography-tandem mass spectrometry using stable isotope dilution. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2003;794:115-123.
9 Putnam SK, Lopata C, Fox JD, Thomeer ML, Rodgers JD, Volker MA, et al. Comparison of Saliva Collection Methods in Children with High-Functioning Autism Spectrum Disorders: Acceptability and Recovery of Cortisol. *Child Psychiatry Hum Dev.* 2012.
10 Bacon CJ, Mucklow JC, Saunders A, Rawlins MD, Webb JK. A method for obtaining saliva samples from infants and young children. *Br J Clin Pharmacol.* 1978;5:89-90.
11 Marchei E, Farre M, Pardo R, Garcia-Algar O, Pellegrini M, Pacifici R, et al. Correlation between methylphenidate and ritalinic acid concentrations in oral fluid and plasma. *Clin Chem.* 2010;56:585-592.
12 Newton R, Broughton LJ, Lind MJ, Morrison PJ, Rogers HJ, Bradbrook ID. Plasma and salivary pharmacokinetics of caffeine in man. *Eur J Clin Pharmacol.* 1981;21:45-52.
13 Zylber-Katz E, Granit L, Levy M. Relationship between caffeine concentrations in plasma and saliva. *Clin Pharmacol Ther.* 1984;36:133-137.
14 Haeckel R. Relationship between intraindividual variation of the saliva/plasma- and of the arteriovenous concentration ratio as demonstrated by the administration of caffeine. *J Clin Chem Clin Biochem.* 1990;28:279-284.
15 Edwards AY, Skolnik JM, Dombrowsky E, Patel D, Barrett JS. Modeling and simulation approaches to evaluate pharmacokinetic sampling contamination from central venous catheters in pediatric pharmacokinetic studies of actinomycin-D: a report from the children's oncology group. *Cancer Chemother Pharmacol.* 2012;70:83-94.
16 Gorodischer R, Koren G. Salivary excretion of drugs in children: theoretical and practical issues in therapeutic drug monitoring. *Dev Pharmacol Ther.* 1992;19:161-177.
17 Haeckel R, Hanecke P. Application of saliva for drug monitoring. An in vivo model for transmembrane transport. *Eur J Clin Chem Clin Biochem.* 1996;34:171-191.
18 Marchei E, Farre M, Pardo R, Garcia-Algar O, Pellegrini M, Pacifici R, et al. Correlation between methylphenidate and ritalinic acid concentrations in oral fluid and plasma. *Clin Chem.* 2010;56:585-592.
19 van GJ, Cohen A. Vanishing clinical psychopharmacology. *Br J Clin Pharmacol.* 2011;72:1-5.
20 Zoethout RW, Delgado WL, Ippel AE, Dahan A, van Gerven JM. Functional biomarkers for the acute effects of alcohol on the central nervous system in healthy volunteers. *Br J Clin Pharmacol.* 2011;71:331-350.
21 de Visser SJ, van der Post JP, de Waal PP, Cornet F, Cohen AF, van Gerven JM. Biomarkers for the effects of benzodiazepines in healthy volunteers. *Br J Clin Pharmacol.* 2003;55:39-50.
22 de Visser SJ, van der Post J, Pieters MS, Cohen AF, van Gerven JM. Biomarkers for the effects of antipsychotic drugs in healthy volunteers. *Br J Clin Pharmacol.* 2001;51:119-132.
23 de Visser SJ, van der Post JP, de Waal PP, Cornet F, Cohen AF, van Gerven JM. Biomarkers for the effects of benzodiazepines in healthy volunteers. *Br J Clin Pharmacol.* 2003;55:39-50.
24 Dumont GJ, de Visser SJ, Cohen AF, van Gerven JM. Biomarkers for the effects of selective serotonin reuptake inhibitors (SSRIS) in healthy subjects. *Br J Clin Pharmacol.* 2005;59:495-510.
25 Zuurman L, Ippel AE, Moin E, van Gerven JM. Biomarkers for the effects of cannabis and THC in healthy volunteers. *Br J Clin Pharmacol.* 2009;67:5-21.
26 Dumont GJ, Verkes RJ. A review of acute effects of 3,4-methylenedioxymethamphetamine in healthy volunteers. *J Psychopharmacol.* 2006;20:176-187.
27 Oettinger L, Jr., Majovski LV. Methylphenidate: a review. *South Med J.* 1976;69:161-163.
28 Pietrzak RH, Mollica CM, Maruff P, Snyder PJ. Cognitive effects of immediate-release methylphenidate in children with attention-deficit/hyperactivity disorder. *Neurosci Biobehav Rev.* 2006;30:1225-1245.
29 Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev.* 1999;51:83-133.
30 Zhang L, Weiner JL, Carlen PL. Potentiation of gamma-aminobutyric acid type A receptor-mediated synaptic currents by pentobarbital and diazepam in immature hippocampal CA1 neurons. *J Pharmacol Exp Ther.* 1993;266:1227-1235.
31 Rhee JS, Jin YH, Akaike N. Developmental changes of $GABA_A$ receptor-chloride channels in rat Meynert neurons. *Brain Res.* 1998;779:9-16.
32 Moy SS, Duncan GE, Knapp DJ, Breese GR. Sensitivity to ethanol across development in rats: comparison to [3H]zolpidem binding. *Alcohol Clin Exp Res.* 1998;22:1485-1492.
33 Chudomel O, Herman H, Nair K, Moshe SL, Galanopoulou AS. Age- and gender-related differences in $GABA_A$ receptor-mediated postsynaptic currents in GABAergic neurons of the substantia nigra reticulata in the rat. *Neuroscience.* 2009;163:155-167.
34 Taketo M, Yoshioka T. Developmental change of GABA(A) receptor-mediated current in rat hippocampus. *Neuroscience.* 2000;96:507-514.
35 Wall MJ. Alterations in $GABA_A$ receptor occupancy occur during the postnatal development of rat Purkinje cell but not granule cell synapses. *Neuropharmacology.* 2005;49:596-609.
36 Hay JL, van Gerven JMA. A meta-analysis of pharmacodynamic testing with the NeuroCart used in the early phase drug development of antidepressants, stimulants, and CNS depressant agents. Dutch Medicines Days, Lunteren, The Netherlands. 2012.
37 Vinkers CH, Klanker M, Groenink L, Korte SM, Cook JM, Van Linn ML, et al. Dissociating anxiolytic and sedative effects of $GABA_A$ergic drugs using temperature and locomotor responses to acute stress. *Psychopharmacology (Berl).* 2009;204:299-311.
38 Kurz A, Sessler DI, Annadata R, Dechert M, Christensen R, Bjorksten AR. Midazolam minimally impairs thermoregulatory control. *Anesth Analg.* 1995;81:393-398.
39 Spoerke N, Underwood S, Differding J, Van P, Sambasivan C, Shapiro D, et al. Effects of ethanol intoxication and gender on blood coagulation. *J Trauma.* 2010;68:1106-1111.
40 Wening K, Breitkreutz J. Oral drug delivery in personalized medicine: unmet needs and novel approaches. *Int J Pharm.* 2011;404:1-9.
41 Roberts M, Vellucci D, Mostafa S, Miolane C, Marchaud D. Development and evaluation of sustained-release Compritol(R) 888 ATO matrix mini-tablets. *Drug Dev Ind Pharm.* 2012;38:1068-1076.
42 WHO statement. 2009. World Health Organization.

43 van Riet-Nales DA, de Neef BJ, Schobben AF, Ferreira JA, Egberts TC, Rademaker CM. Acceptability of different oral formulations in infants and preschool children. *Arch Dis Child.* 2013;98:725-731.
44 Chernik DA, Gillings D, Laine H, Hendler J, Silver JM, Davidson AB, et al. Validity and reliability of the Observer's Assessment of Alertness/Sedation Scale: study with intravenous midazolam. *J Clin Psychopharmacol.* 1990;10:244-251.
45 Lamas A, Lopez-Herce J. Monitoring sedation in the critically ill child. *Anaesthesia.* 2010;65:516-524.
46 Link B, Haschke M, Grignaschi N, Bodmer M, Aschmann YZ, Wenk M, et al. Pharmacokinetics of intravenous and oral midazolam in plasma and saliva in humans: usefulness of saliva as matrix for CYP3A phenotyping. *Br J Clin Pharmacol.* 2008;66:473-484.
47 Davies FC, Waters M. Oral midazolam for conscious sedation of children during minor procedures. *J Accid Emerg Med.* 1998;15:244-248.
48 Kapogiannis BG, Mattison DR. Adolescents in clinical trials. *Clin Pharmacol Ther.* 2008;84:655-659.
49 Kennedy M. Hormonal regulation of hepatic drug-metabolizing enzyme activity during adolescence. *Clin Pharmacol Ther.* 2008;84:662-673.
50 Everett AV. Pharmacologic treatment of adolescent depression. *Curr Opin Pediatr.* 2002;14:213-218.
51 Andersen SL. Trajectories of brain development: point of vulnerability or window of opportunity? *Neurosci Biobehav Rev.* 2003;27:3-18.
52 Andersen SL, Navalta CP. Altering the course of neurodevelopment: a framework for understanding the enduring effects of psychotropic drugs. *Int J Dev Neurosci.* 2004;22:423-440.
53 Spetie L, Arnold LE. Ethical issues in child psychopharmacology research and practice: emphasis on preschoolers. *Psychopharmacology (Berl).* 2007;191:15-26.
54 Greenhill L, Kollins S, Abikoff H, McCracken J, Riddle M, Swanson J, et al. Efficacy and safety of immediate-release methylphenidate treatment for preschoolers with ADHD. *J Am Acad Child Adolesc Psychiatry.* 2006;45:1284-1293.
55 Graham J, Coghill D. Adverse effects of pharmacotherapies for attention-deficit hyperactivity disorder: epidemiology, prevention and management. CNS *Drugs.* 2008;22:213-237.
56 Wigal T, Greenhill L, Chuang S, McGough J, Vitiello B, Skrobala A, et al. Safety and tolerability of methylphenidate in preschool children with ADHD. *J Am Acad Child Adolesc Psychiatry.* 2006;45:1294-1303.
57 Yuill K, Carandang C. Safety methodology in pediatric psychopharmacology trials. *J Child Adolesc Psychopharmacol.* 2013;23:148-162.
58 Carlton JE. Core Curriculum for Professional Education in Pain. 2005. Seattle, WA, USA, IASP Press.
59 Connelly MA, Brown JT, Kearns GL, Anderson RA, St Peter SD, Neville KA. Pupillometry: a non-invasive technique for pain assessment in paediatric patients. *Arch Dis Child.* 2014.
60 Anderson BJ, Woollard GA, Holford NH. Acetaminophen analgesia in children: placebo effect and pain resolution after tonsillectomy. *Eur J Clin Pharmacol.* 2001;57:559-569.
61 Lavigne JV, Schulein MJ, Hahn YS. Psychological aspects of painful medical conditions in children. I. Developmental aspects and assessment. *Pain.* 1986;27:133-146.
62 Anseloni VC, Weng HR, Terayama R, Letizia D, Davis BJ, Ren K, et al. Age-dependency of analgesia elicited by intraoral sucrose in acute and persistent pain models. *Pain.* 2002;97:93-103.
63 Opsommer E, Plaghki L. Maturational changes in the thermoalgesic system in humans from childhood to adulthood revealed by CO(2) laser evoked brain potentials following cutaneous heat stimuli. *Neurosci Lett.* 2001;316:137-140.

Summary

This thesis describes the potential role of non-invasive measurement of pharmacokinetics (PK) and pharmacodynamics (PD) in the research and development of central nervous system (CNS) stimulants or depressants for children and adolescents. First, we evaluated the feasibility of using saliva as an alternative to plasma in two studies on psychostimulants (caffeine and methylphenidate). Second, neuropsychological and neurophysiological functions were measured longitudinally using the NeuroCart, a battery of tests developed at the Centre for Human Drug Research (CHDR, Leiden, The Netherlands) that includes non-invasive tests for alertness, visuomotor coordination, motor control, memory, and subjective drug effects. Using a non-invasive approach, age-dependent differences in alcohol PK and PD were evaluated between healthy adolescents and adults. This thesis concludes with the report of two clinical trials that were designed to evaluate age-appropriate formulations of sedative drugs that have the potential for use in children.

CHAPTER 1: INTRODUCTION

The pharmacological treatment of children and adolescents with diseases of the CNS has traditionally followed the development program in adults. However, because of differences in neuropsychopathology and development-specific differences in PK and/or PD, the relationships between drug action and drug exposure in children cannot be understood fully by simply extrapolating information from adult patients. Therefore, it is important to investigate age-dependent differences in PK and PD. Unfortunately, our understanding of these differences is based almost entirely on animal models. In addition, the registration of CNS drugs for use in children and adolescents has lagged behind new developments in adults. The situation has been complicated even further by a decrease in the number of new drug registrations for psychiatric and neurological indications in adults.

Despite these factors, the number of treated children and adolescents, as well as the duration of exposure to CNS drugs, has increased substantially over the past few decades. Recent European legislation (the EU Pediatric Regulation) will likely drive an increase in pediatric trials and specific label changes, dosing recommendations, and age-appropriate formulations. Several challenges have emerged when working within the framework of this new legislation and there is an urgent need for validated assessment tools that are suitable for evaluating the efficacy and safety of CNS drugs in the pediatric population. In addition, researchers should attempt to reduce the burden placed on participating children and adolescents by using non-invasive or minimally invasive measurement methods.

The most accessible and non-invasive means to measure drug activity in the brain is to measure drug-related CNS functional activity using methods that provide sufficient sensitivity and specificity. To relate drug-related changes in CNS functional activity to changes in PK, drug concentrations must be measured. Traditional PK protocols – with multiple samples and indwelling catheters or multiple venipunctures – are undesirable in therapeutic pediatric drug research. To overcome some of these limitations, other sample collection methods for determining drug concentration (for example, saliva sampling) have been developed and validated. Saliva sampling has the added benefit of allowing on-site testing without the need for medical personnel or complicated post-collection sample processing, thereby further decreasing the burden placed on the children. Unfortunately, however, the usefulness of determining the saliva concentration of several drugs has been questioned because of variability in the saliva:plasma concentration (S/P) ratio. We propose that if the sources of variability in the S/P ratio can be quantified or minimized, measuring the saliva drug concentration might be a meaningful alternative to measuring plasma drug concentration.

CHAPTER 2: THE EUROPEAN PEDIATRIC REGULATION: WILL IT PROVIDE CHILDREN WITH THE MEDICINES THEY NEED?

In this chapter, a study is described in which the impact of the European Pediatric Regulation on the development of pediatric medicines – including CNS drugs – is evaluated. The Regulation requires the pharmaceutical industry to plan clinical trials in children in an early stage during drug development in adults or in case a new indication, formulation or administration route is investigated for adults for on-patent medicines. The so-called Pediatric

Investigational Plan (PIP) describes how a medicine should be investigated in children. This plan should be presented by the company early in the development of a medicine and subsequently agreed with the Pediatric Committee of the EMA. In this study, we evaluated the drug classes for which pediatric development was either agreed for development or was waived by the EMA from 2007 until March 2012. In addition, we evaluated whether the Regulation is likely to lead to the development of drug classes for which there exists a (unmet) pediatric need, or for which pharmaceutical expenditure is high. In addition, Dutch physicians working in pediatric healthcare indicated if they find that the pediatric medicines (developed and researched under the Regulation) are actually needed.

From 2007 until March 2012, approximately two-thirds of the medicines were agreed by the EMA for pediatric development; deferral of the start or completion of measures in the PIP until after authorization for adults was granted for 83% percentage of these medicines. Drug classes and therapeutic subgroups with a high need for pediatric research and development on the EMA Needs Lists, like CNS drugs, are either researched infrequently or often waived from pediatric development. In addition, medicines that are frequently prescribed (PHARMO Database Network, 2005-2011), but are not always readily available (and that therefore represent an unmet pediatric need) are researched relatively rarely under the Regulation. The drug classes in our evaluation with the lowest number of medicines with agreed pediatric development had the lowest pharmaceutical expenditure (GIP databank, 2007-2011). Finally, fifty Dutch physicians working in pediatric healthcare were not convinced that medicines for which pediatric development was agreed are needed for their clinical practice. Our analysis confirms the increased need for researching and developing CNS drugs for use in pediatric patients, as drugs acting on the nervous system (the so-called neurologicals) had the highest number of off-label active substances for which the EMA Needs Lists identified a need[2]. Nearly half of all neurologicals listed were indicated to need full pediatric development. Anesthetics, analgesics, antiepileptics, and psycholeptics (such as antipsychotics, anxiolytics, hypnotics, and sedatives) were among the therapeutic subgroups with the highest number of medicines with a pediatric need. Our analysis also indicates that the percentage and number of children and adolescents who are treated using CNS drugs continues to rise, reflected by an annual increase in out-patient use of neurologicals in Dutch children and adolescents from 2005 through 2011 (the end of the study period).

We conclude that the Regulation's key strategy does not necessarily lead to the increased pediatric development of drug classes for which there may be a (unmet) pediatric need. Instead, the Regulation's output is in line with expenditure data, most likely as a result of the 'adult-driven' approach. In addition, given the high number of granted deferrals it is likely that the registration of CNS drugs for use in children and adolescents still lags behind new developments in adults. We propose that important refinements in implementation are needed in order to ensure that the Regulation will provide children and adolescents with the medicines they actually need. For example, evaluation of any proposal for a PIP (or request for a waiver) should be based on the potential pediatric relevance of the mechanism of action or drug target, and new incentives should be considered for first-in-children indications.

CHAPTER 3: BIOMARKERS OF ACUTE METHYLPHENIDATE EFFECTS IN CHILDREN AND ADOLESCENTS WITH ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

The psychostimulant methylphenidate (MPH) is the most commonly prescribed medication for treating pediatric attention-deficit/hyperactivity disorder (ADHD). Previously published studies investigating the effects of immediate-release MPH (MPH-IR) yielded contradictory results due to several sources of variability, for example a lack of standardized biomarkers for drug-effect measurements.

In the study described in chapter 3, we performed a systematic literature review to identify sensitive and useful non-invasive biomarkers for monitoring the effects of MPH-IR in children and adolescents with ADHD. We identified 78 randomized placebo-controlled clinical studies (published until December 2009) that investigated CNS effects following a single dose of MPH-IR in pediatric ADHD patients. Outcome measures were clustered to groups of related

tests or test variants (referred to as 'clusters') in order to generate a reasonable degree of standardization across studies and tests. We performed a progressive condensation of the results into logical clusters, thus providing a more general assessment of the drug's effects on groups of comparable tests or functional domains. Neurocognitive clusters and individual tests that were used in five or more studies were evaluated for reporting consistent MPH effects.

The results of our review revealed that a wide variety of biomarkers are currently being used to evaluate the effect of MPH-IR in ADHD. The following outcomes showed a consistent response to a therapeutic MPH dose across studies based on different cohorts: Continuous Performance Test, Go/no-go Task, Visual Evoked Potentials, and several observation scales (including Following Rules Observations, Oppositional Behavior Observations, On-Task Behavior Observations, and Impulsivity Behavior Observations). MPH's effect was best detected in tests and observations regarding motor control, sustained attention, divided attention, and impulsivity (inhibitory control), indicating that MPH has acute effects on all three core symptoms of ADHD (inattention, hyperactivity, and impulsivity) among MPH-responsive children with ADHD.

We propose that the potential biomarkers identified in this review might help identify responders versus non-responders following a test dose of MPH. Because dose-effect relationships could not be quantified, these tests and clusters should be investigated further in order to thoroughly evaluate the dose-response relationships, including effect size, and establish clinically relevant changes. Ideally, these studies should include concentration- (in addition to dose-) effect relationships at several time points in order to profile the effect of MPH treatment in children and adolescents with ADHD.

CHAPTER 4: DETERMINATION OF METHYLPHENIDATE IN PLASMA AND SALIVA BY LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

Monitoring MPH concentrations can help determine whether a lack of observed efficacy and/or the presence of unexpected adverse effects are related to PK or PD factors. In adults, clinical monitoring of MPH therapy is usually performed by measuring plasma MPH concentrations. In children, however, blood sampling is undesirable. Saliva may be an alternative matrix for monitoring MPH concentrations; however, several potential complicating factors have been encountered in previous studies. Complicating factors included indications of oral contamination in the first few saliva samples after taking MPH tablets and considerable – yet unexplained – variation in the S/P ratio throughout the time course of both tablet and capsule formulations.

Obtaining an accurate S/P ratio is essential for realizing the full potential of using saliva sampling to monitor plasma MPH concentrations. Therefore, we developed an analytical method for accurate and precise quantification of MPH in both plasma and saliva. In this chapter, we present the validation of a liquid chromatography – tandem mass spectrometric method using a hydrophilic interaction liquid chromatography column (HILIC). In a 100 µl sample, proteins were precipitated with 750 µl acetonitrile/methanol 84/16 (v/v) containing d_9-methylphenidate as the internal standard. Standard curves were prepared over the MPH concentration range of 0.5 – 100.0 µg/L. The total analysis time was 45 seconds. Accuracy and within- and between-run imprecision were in the range of 98-108% and less than 7.0%, respectively. Matrix effects were greater for plasma than saliva with 46% and 8% ionization suppression. The matrix effects were adequately compensated by the use of deuterated MPH as internal standard. MPH significantly degraded in plasma and saliva at room temperature and 5°C. Stability experiments demonstrated that samples should be stored at temperatures of -20°C or below directly after sampling, and that samples should be processed immediately after thawing. Samples were stable at -20°C for at least 4 weeks. The method was successfully applied for the determination of MPH concentrations in plasma and saliva samples from an adult healthy volunteer.

We conclude that using protein precipitation and hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry, this method allows fast, accurate and precise quantification of MPH in both plasma and saliva.

CHAPTER 5: POPULATION PHARMACOKINETICS MODELING OF TWO METHYLPHENIDATE FORMULATIONS IN PLASMA AND SALIVA OF HEALTHY SUBJECTS

In this chapter, a study is described in which a first attempt was made to quantify sources of variability in MPH plasma and saliva concentrations, and to describe the relationship between MPH concentration in saliva and MPH concentration in plasma using a population PK modeling approach.

In this randomized, open-label study, MPH-IR (tablet) and osmotic release oral system MPH (MPH-OROS, capsule) were administered in a crossover design to 12 healthy adult subjects (six men and six women). Paired blood and saliva samples were collected pre-dose and at regular intervals for 6 (MPH-IR) or 11 (MPH-OROS) hours following drug administration. Population PK analysis was performed using nonlinear mixed-effect modeling.

A one-compartmental structure model with first-order absorption (with separate compartments for MPH-IR and MPH-OROS) and first-order elimination provided the best description of estimated MPH plasma PK. The estimated clearance was 6.0 liters/hour and the volume of distribution was 7.5 liters. The derived terminal half-life was 0.9 hours. Inter-individual variability was identified on clearance, the volume of distribution, and the absorption rate constant for MPH-OROS. The S/P ratio was 2.44 from 2.5 hours onward. Inter-individual variability was identified in the S/P ratio.

With proper allometric scaling techniques, we expect that this PK model can used in children to predict the concentration-time profile in the plasma using MPH concentrations measured in saliva samples. Further studies are needed to determine the predictive performance of the model in children with ADHD.

CHAPTER 6: CAFFEINE PHARMACOKINETICS AND EFFECTS ON CENTRAL AND AUTONOMOUS NERVOUS SYSTEM PARAMETERS IN ADOLESCENTS

Children and adolescents frequently use caffeine as a psychostimulant. Despite prevalent use of caffeine among adolescents, remarkably little research has been conducted regarding the physiological and behavioral effects of caffeine in this age group. Data obtained from animal studies suggest that the effects of caffeine reported in adults cannot be extrapolated simply to adolescents.

Therefore, in chapter 6, we evaluated the effect profile of caffeine on central and autonomic nervous system parameters following the consumption of a low dose caffeinated beverage by healthy adolescents; the results were compared with data obtained following the consumption of a non-caffeinated beverage. Caffeine concentrations were measured from saliva samples. In a separate study using adult volunteers, we determined the extent of oral contamination with caffeine after consuming a caffeinated beverage versus swallowing a caffeine capsule (200 mg). Both saliva and plasma samples were collected simultaneously in order to measure the S/P ratio of caffeine concentration. Based on the data collected from this kinetic study, a population PK model was built to estimate plasma drug levels in adolescents; this model could be used to develop a pharmacokinetic-pharmacodynamic (PK/PD) model.

In adolescents, caffeine had significant effects on task parameters related to attention and visuomotor coordination (adaptive tracking task) and alertness (saccadic peak velocity). In addition, an increase in error rate in the attention switch task was observed after caffeine. Plasma caffeine concentrations in adults were described best as a two-compartment model with a dose depot, first-order absorption kinetics, and first-order elimination kinetics. The plasma model identified a dose of 90 mg in the caffeinated beverage. Lean body mass-dependent variability was identified for the volume of the central compartment. This PK model was expanded to a population model

that described saliva caffeine concentrations in adults >1 hour after administration as a fraction (0.68) of plasma concentration (i.e., the S/P ratio was 0.68). Before 1 hour after administration, saliva caffeine concentrations could not be described as a linear fraction; therefore, caffeine's early effects in adolescents were not suitable for inclusion in a PK/PD model.

We conclude that in healthy, alert adolescents, 90 mg caffeine has significant effects on parameters regarding alertness and reaction speed, despite the relatively low dose and the expected ceiling effect in this healthy and alert population. Whether these effects observed in adolescents are larger in adolescents than in adults remains to be determined.

CHAPTER 7: COMPARISON OF THE PHARMACOKINETICS AND EFFECTS OF ALCOHOL ON OBJECTIVE AND SUBJECTIVE BIOMARKERS BETWEEN HEALTHY ADOLESCENTS AND ADULTS

Our understanding of age-dependent differences in PK and PD of CNS drugs is based almost entirely on animal models. In this study, we used a PK/PD modeling approach to compare the objective and subjective responses to alcohol between adolescent and adult subjects. The acute effect of consuming a socially accepted dose of alcohol (two standard units) was determined in 16-18-year-old adolescents. Blood alcohol concentration was measured non-invasively using end-expired breath samples. A PK/PD model was then developed by combining the data obtained from this study in adolescents with data obtained from previous alcohol studies (using the clamping method) performed in adults. This model was used to characterize alcohol's PK and effects on an objective biomarker and a subjective biomarker and to explore potential sources of variability, including age.

A two-compartment structural model with first-order absorption and Michaelis-Menten elimination provided the best description of estimated plasma alcohol PK. Inter-individual variability was identified for several kinetics parameters, with lean body weight-dependent variability in peripheral compartment volume and maximum elimination, weight-dependent variability in central compartment volume, and height-dependent and age-dependent variability in intercompartment clearance.

Smooth pursuit performance and VAS Alertness were selected as biomarkers for PK/PD modelling based on an exploratory meta-analysis of all relevant alcohol data. The relationship between alcohol concentration and the effects of alcohol on baseline smooth pursuit performance and VAS Alertness score was described best as being dose-dependent, with no indications of delay or tolerance. Higher baseline performance for smooth pursuit was correlated with a larger absolute decrease in performance. No covariates were identified for the relationship between alcohol concentration and effect with respect to smooth pursuit performance or VAS Alertness score.

The covariates that were identified in our PK model may be (in)directly related to differences between adolescents and adults, as considerable age-related and maturity-related changes in body composition occur during adolescence. VAS Alertness and smooth pursuit eye movements may not necessarily represent all of the alcohol-related effects on the CNS, and we cannot exclude the possibility that sensitivity to other alcohol-related pharmacodynamics effects change with age. Unfortunately, other sensitive functional biomarkers for alcohol effects were less suitable for developing a PK/PD model, as the small effect in adolescents and the high number of non-responders precluded our ability to quantify the adolescent data and evaluate an age-dependent effect. Therefore, whether sensitivity to other alcohol-related pharmacodynamics effects changes with age remains to be determined.

CHAPTER 8: PHARMACOKINETICS OF PROLONGED-RELEASE MELATONIN MINI-TABLETS IN CHILDREN WITH BOTH AUTISM SPECTRUM DISORDER AND A SLEEP DISORDER

Melatonin is one of the CNS drugs identified by the EMA as having a pediatric therapeutic need. This includes the need to develop an age-appropriate sustained-release formulation and the need to collect data regarding melatonin's

PK, efficacy, and safety in children with autism spectrum disorder (ASD) and a sleep disorder. The study described in chapter 8 is part of a Pediatric Investigation Plan (PIP) under the Pediatric Regulation. This was a cross-over ascending dose study of Circadin (1 mg 3-mm diameter mini-tablets), a prolonged-release melatonin formulation. In this study, the PK profile, safety, and acceptability of Circadin were evaluated in 16 children and adolescents with autism and a sleep disorder. We tested 2-mg and 10-mg doses of Circadin based on the dose range we will use in an upcoming efficacy trial, which is also part of the development plan. To minimize the number of samples taken during the night, Circadin was administered in the early morning. The first occasion included a 24-hour baseline measurement day. Whole-saliva samples were collected non-invasively from passive drool, and melatonin concentration was measured. Urine samples were collected for determination of the metabolite 6-sulphatoxymelatonin (6-SMT). Adverse events were monitored throughout the study, and sedative effects were assessed using the Observer's Assessment of Alertness/Sedation (OAA/S) scale for 10 hours after administration. PK parameters for melatonin were estimated using non-compartmental modeling.

All 16 subjects (12 male, 4 female; age range: 7-15 years) had a clinical diagnosis of autism spectrum disorder (based on DSM-IV-TR criteria). All reported side effects were consistent with known side effects. Mini-tablets were found to be both safe (i.e., none of the children choked) and acceptable to the children. The melatonin concentration peaked within two hours of administration and remained elevated for several hours thereafter. Circadin exposure was dose-linear, and clearance (1,000 L/hr) was similar between the dose groups. The median apparent terminal half-life was comparable between dosages. The mean total 6-SMT recovered from urine during the baseline period was 4.2 µg/12 daytime hours and 13.5 µg/12 nighttime hours. Following administration with Circadin 2 mg, the mean amount of total 6-SMT recovered from urine was 989.5 µg/12 daytime hours and 95.3 µg/12 nighttime hours. The highest levels of sedation (assessed using the OAA/S) were observed between 2 and 3 hours after administration of Circadin 2 mg and between 2 and 6 hours after administration of Circadin 10 mg. Overall, the subjects and their caregivers were positive about the burden and duration of the study. Nearly all subjects (69%) and caregivers (88%) stated that they would consider (consent for) participating in a similar trial again.

We conclude that this study demonstrates the short-term safety, acceptability, and prolonged-release profile of Circadin mini-tablets in 16 school-age children and adolescents with ASD.

CHAPTER 9: PHARMACOKINETICS AND PHARMACODYNAMICS OF A NEW HIGHLY CONCENTRATED INTRANASAL MIDAZOLAM FORMULATION FOR CONSCIOUS SEDATION

Because of its rapid onset and rapid recovery profile, midazolam is the medication of choice for providing conscious sedation and management of epileptic seizures. Nasal delivery of midazolam is a non-invasive alternative to intravenous administration. However, previous formulations for delivering midazolam nasally have not been very successful due to the lack of solvents that can dissolve midazolam at therapeutic dosages without causing nasal mucosa damage.

In the study described in chapter 9, the pharmacokinetics of two doses of a novel highly concentrated aqueous intranasal midazolam formulation (Nazolam) was characterized. In this four-way crossover, double-blind, double-dummy, randomized, placebo-controlled study, 16 subjects received 2.5 mg Nazolam, 5.0 mg Nazolam, 2.5 mg intravenous midazolam or placebo on different occasions. Pharmacokinetics of midazolam and α-hydroxy-midazolam were characterized and related to outcome variables for sedation (saccadic peak velocity, the Bond and Lader Visual Analogue Scale for sedation, the simple reaction time task and the Observer's Assessment of Alertness/Sedation). The onset and duration of the pharmacological effect were evaluated and compared to intravenous midazolam using the biomarker saccadic peak velocity (SPV), as a relationship between SPV reduction and clinical efficacy has been described. Nasal tolerance was evaluated through subject reporting and ENT examination.

Bio-availability of Nazolam was 75%. Maximal plasma concentrations of 31 ng/ml (CV, 42.3%) were reached after 11 minutes (2.5 mg Nazolam), and of 66 ng/ml (CV, 31.5%) after 14 minutes (5.0 mg Nazolam). Sedation onset (based on -2SD SPV change) occurred 1 minute after administration of 2.5 mg intravenous midazolam, 7 minutes after 2.5 mg Nazolam, and 4 minutes after 5 mg Nazolam. Sedation duration was 85 minutes for 2.5 mg intravenous midazolam, 47 minutes for 2.5 mg Nazolam, and 106 minutes for 5.0 mg Nazolam. clinically relevant levels of sedation (as measured using OAA/S) were achieved within minutes after administration. In addition, single administration was well tolerated and safe, and did not lead to nasal mucosa damage.

We conclude that this study demonstrates the nasal tolerance, short-term safety and efficacy of this novel formulation in 16 healthy adult subjects. When considering the preparation time needed for obtaining venous access, conscious sedation can be achieved in the same time span as needed for intravenous midazolam. This non-invasive formulation may offer important advantages in conscious sedation and epilepsy and has potential for use in children.

CHAPTER 10: GENERAL DISCUSSION

CNS drugs are researched infrequently under the Regulation, despite the high need for pediatric research and development, and despite the ongoing increase in the use of these medicines among pediatric patients. Based on our data, it is unlikely that the Regulation will have a positive impact on the ability of children and adolescents to access new CNS drugs (as only few new CNS drugs are developed for adults), or on the delay in pediatric registration of CNS drugs as described in the Introduction to this thesis. Given the high number of CNS drugs with deferred pediatric studies, clinical trial strategies should be revised in a timely fashion.

In the Introduction to this thesis, we proposed that if variability in the S/P ratio can be minimized or quantified, measuring the saliva drug concentration might be a meaningful alternative (to measuring plasma drug concentration) for more drug types than has seemed feasible until now. The studies described in chapters 5 and 6 attempted to investigate and quantify the sources of this variability using a population PK modeling approach. We showed that the relationship between plasma and saliva concentrations of MPH is stable beyond 2.5 hours and of caffeine beyond one hour after administration. Prior to these time points, the relationship between plasma and saliva concentrations was not linear; unfortunately, our efforts to correct for factors that potentially contribute to variability in the S/P ratio were unsuccessful. The number of saliva samples that can be collected to measure the absorption phase of compounds with a short T_{max} (such as MPH and caffeine) is limited, thus hampering the ability to determine the magnitude of contamination, the S/P ratio, and sources of variability in the S/P ratio directly following administration. For these drug types, only CNS effects that occur relatively late are suitable for developing a PK/PD model using measured saliva concentrations or predicted plasma concentrations. However, because the pharmacological CNS action of most compounds is delayed relative to changes in plasma concentration, saliva sampling may still be a viable option for this type of research. Therefore, our results support the further exploration of using saliva as a non-invasive method for drug profiling CNS drugs.

The non-invasive CNS tests that are included in the NeuroCart battery are sensitive enough to detect effects of low doses of caffeine and alcohol in healthy adolescents, as shown by the studies described in chapters 6 and 7. Significant effects on parameters regarding alertness (saccadic peak velocity) and reaction time (adaptive tracking) were observed after caffeine was administered, despite the relatively low dose and the expected ceiling effect on several parameters in this cohort of healthy, alert adolescents. Because caffeine has been reported to produce behavioral effects (including motor activation and arousal) similar to the effect of classic psychostimulants such as cocaine and amphetamine, we proposed that these tasks could be valuable in pediatric studies using other CNS stimulants. In the study described in chapter 7, a low dose of alcohol induced significant changes in smooth pursuit eye movements, VAS Alertness score, the VAS alcohol effect score, body sway, systolic blood pressure, and heart rate. Animal studies have revealed

developmental changes in the pharmacological sensitivity of $GABA_A$-receptor-mediated currents to several drugs. Therefore, these tasks could be used to study age-related differences in the CNS effects of GABA-ergic compounds in pediatric drug research. Importantly, the CNS tests and their duration were tolerated by the majority of adolescent subjects in these clinical studies.

In the Introduction to this thesis, we raised several issues with respect to pediatric neuropsychopharmacology. First, the differences in neuropsychopathology and pharmacology between children and adults must be recognized. In addition, researchers need validated tools that are appropriate for assessing the efficacy and safety of CNS drugs in children. Finally, special emphasis should be placed on formulation research. We propose that CNS drug profiling has the potential to address all of these issues. First, CNS drug profiling enables researchers to evaluate age-dependent effects by drawing comparisons with existing data collected in adults, as demonstrated by the study described in chapter 7. Second, several drug classes have a unique CNS drug profile on the NeuroCart test battery, which corresponds to the drug class' mechanism of action. We have demonstrated previously that these "fingerprints' can be used to differentiate a drug's stimulant and sedative properties, for example by evaluating effects on saccadic peak velocity. As mentioned already, the CNS tests that are included in the NeuroCart battery are sensitive enough to detect low doses of caffeine and alcohol in adolescents. Functional biomarkers could be used to determine whether a specific drug exerts a pharmacological effect at a specific dose in children, and they could also be used to monitor (un)wanted sedative or stimulant properties of drugs in children. Third, non-invasive monitoring of PK and PD could facilitate the evaluation of age-appropriate formulations of CNS drugs for use in children, as illustrated by the clinical studies described in chapters 8 and 9. The 'proof-of-pharmacology' strategy described in chapter 9 may perhaps also be followed in pediatric studies on drugs for which extensive data exist on efficacy and safety, but for which there is only a need for an age-appropriate formulation.

In parallel with further refining the methods described in this thesis, several other steps must be taken. First, non-invasive drug monitoring should be evaluated in younger age groups and in subjects with other neuropsychiatric disorders. A second important step, particularly considering that most neurological and psychiatric disorders are either chronic or recurrent, is to determine whether early treatment translates into an acute improvement in symptom-related CNS functions as well as improved long-term outcome. Finally, as there is a need to study the effects of anesthetics and analgesics in children and adolescents, more experience should be gained in the field of pediatric pain research.

CONCLUSION

The clinical studies included in this thesis show that non-invasive drug profiling for CNS stimulants or depressants is feasible in healthy adolescents and children and adolescents with ASD. In addition, non-invasive drug profiling may help researchers both evaluate age-dependent PK and PD and compare the effect profiles of various formulations. Importantly, this approach may also facilitate the execution of studies included in Pediatric Investigation Plans under the Pediatric Regulation, thus representing an important step forward, particularly given the high need for pediatric research with respect to CNS drugs. The ideal pediatric study should be executable in the subjects' homes. Adult studies of novel drugs that could potentially be measured in saliva samples should include measurements of the S/P concentration ratio. Because considerable PK variability has been reported for several neuropsychiatric drugs, sensitive non-invasive or minimally invasive PD measurements should be assessed longitudinally. In addition, pediatric studies should ideally include a means to compare the results with adult studies. Finally, to expand our knowledge regarding the acceptability and tolerability of measurement methods, whenever possible the study participants should be asked to complete a questionnaire in order to collect information regarding the reasons why children and adolescents participate, as well as their perceived burden associated with the study.

Samenvatting
Summary in Dutch

Dit proefschrift beschrijft de mogelijke rol van het non-invasief meten van farmacokinetiek en farmacodynamiek tijdens het onderzoek naar en de ontwikkeling van geneesmiddelen met een stimulerend of onderdrukkend effect op het centrale zenuwstelsel bij kinderen en adolescenten. Allereerst hebben wij in twee studies met psychostimulantia (cafeïne en methylfenidaat) de uitvoerbaarheid van het gebruiken van speeksel als een alternatief voor plasma onderzocht. Ten tweede zijn neuropsychologische en neurofysiologische functies longitudinaal gemeten met de NeuroCart, een testbatterij die door het Centre for Human Drug Research (CHDR, Leiden, Nederland) is ontwikkeld en non-invasieve testen bevat voor alertheid, visuomotore coördinatie, motorische controle, geheugen en subjectieve geneesmiddeleffecten. Gebruikmakend van non-invasieve methoden zijn leeftijdsafhankelijke verschillen tussen gezonde adolescenten en volwassenen in de farmacokinetiek en farmacodynamiek van alcohol onderzocht. Dit proefschrift sluit af met de rapportage van de resultaten van twee klinische studies gericht op het evalueren van leeftijdsgeschikte toedieningsvormen van sedativa die mogelijk bij kinderen toegepast kunnen worden.

HOOFDSTUK 1: INTRODUCTIE

De medicamenteuze behandeling van kinderen en adolescenten met aandoeningen van het centrale zenuwstelsel heeft vanouds het ontwikkelprogramma bij volwassenen gevolgd. Vanwege verschillen in de neuropsychopathologie en ontwikkelingsspecifieke verschillen in de farmacokinetiek en/of farmacodynamiek kan de relatie tussen geneesmiddeleffect en geneesmiddelblootstelling bij kinderen echter niet altijd volledig worden begrepen door extrapolatie van informatie die is verkregen bij volwassen patiënten. Om deze reden is het belangrijk om leeftijdsafhankelijke verschillen in de farmacokinetiek en farmacodynamiek nader te onderzoeken. Helaas is ons begrip van deze verschillen bijna volledig gebaseerd op diermodellen. Bovendien loopt de registratie van geneesmiddelen met een effect op het centrale zenuwstelsel bij kinderen en adolescenten achter bij nieuwe ontwikkelingen bij volwassenen. Deze situatie wordt nog verder gecompliceerd door het dalen van het aantal registraties van nieuwe geneesmiddelen voor psychiatrische en neurologische indicaties bij volwassenen.

Ondanks bovengenoemde factoren is het aantal kinderen en adolescenten dat wordt behandeld met geneesmiddelen met een effect op het centrale zenuwstelsel alsmede de duur van blootstelling aan deze geneesmiddelen de afgelopen decennia fors toegenomen. Recente Europese wetgeving (de '*EU Pediatric Regulation*', de Europese pediatrische Verordening) zal waarschijnlijk leiden tot een toename in het aantal studies bij kinderen en tot specifieke aanpassingen in de productbeschrijving van geneesmiddelen, doseringsadviezen en leeftijdsgeschikte toedieningsvormen. Er zijn verschillende uitdagingen naar voren gekomen tijdens het werken binnen het raamwerk van deze nieuwe wetgeving en er bestaat een dringende behoefte aan gevalideerde meetmethoden voor het evalueren van effectiviteit en veiligheid van geneesmiddelen met een effect op het centrale zenuwstelsel in de pediatrische populatie. Bovendien is het wenselijk dat onderzoekers proberen om de belasting van kinderen en adolescenten tijdens het deelnemen aan een studie te verminderen door gebruik te maken van non-invasieve of minimaal-invasieve meetmethoden.

De meest toegankelijke en non-invasieve manier om een geneesmiddeleffect in de hersenen te meten is door het bepalen van geneesmiddel-gerelateerde functionele activiteit van het centrale zenuwstelsel waarbij gebruik gemaakt wordt van methoden die voldoende sensitief en specifiek zijn. Om deze geneesmiddel-gerelateerde functionele activiteit te relateren aan veranderingen in farmacokinetiek dienen geneesmiddelconcentraties gemeten te worden. Traditionele protocollen voor het meten van de farmacokinetiek – bijvoorbeeld door middel van meerdere afnames en verblijfskatheters of meerdere venapuncties – zijn ongewenst in therapeutisch geneesmiddelonderzoek bij kinderen.

Om een aantal van deze beperkingen te boven te komen, zijn er andere verzamelmethoden voor het bepalen van de geneesmiddelconcentratie ontwikkeld en gevalideerd. Een voorbeeld van een dergelijke verzamelmethode is speekselverzameling. Speekselverzameling heeft het bijkomende voordeel dat het op locatie gedaan kan worden zonder de noodzaak voor medisch-geschoold personeel of gecompliceerde verrichtingen na afname van het monster, waardoor de belasting voor het kind nog verder wordt verminderd.

Helaas is door variabiliteit in de speeksel:plasma concentratie (S/P) ratio het nut van het bepalen van speekselconcentraties van verschillende geneesmiddelen in twijfel getrokken. Wij stellen voor dat wanneer de variabiliteit in de S/P ratio kan worden gekwantificeerd of geminimaliseerd, het meten van speekselconcentraties van een geneesmiddel een zinvol alternatief kan zijn voor het meten van plasmaconcentraties.

HOOFDSTUK 2: DE EUROPESE PEDIATRISCHE VERORDENING: ZAL HET LEIDEN TOT GENEESMIDDELEN DIE KINDEREN NODIG HEBBEN?

In dit hoofdstuk wordt een studie beschreven waarin het effect van de Europese pediatrische Verordening (*European Pediatric Regulation*) is onderzocht op de ontwikkeling van geneesmiddelen voor kinderen, waaronder ook geneesmiddelen met een effect op het centrale zenuwstelsel. De Verordening verplicht farmaceutische bedrijven om klinische studies bij kinderen te plannen tijdens een vroeg stadium van de ontwikkeling van nieuwe geneesmiddelen of in die gevallen waarin een nieuwe indicatie, toedieningsvorm of toedieningsweg wordt onderzocht voor volwassenen voor gepatenteerde geneesmiddelen. Het Pediatrische Onderzoeksplan (*Pediatric Investigational Plan*, PIP) beschrijft hoe een geneesmiddel bij kinderen onderzocht moet worden. Dit plan moet door het bedrijf in een vroeg stadium worden aangeboden aan het Pediatrisch Comité (*Pediatric Committee*, PDCO) van de EMA (*European Medicines Agency*, het Europese Geneesmiddelen Agentschap), die vervolgens het plan beoordeelt. In deze studie hebben wij onderzocht voor welke geneesmiddelklassen de EMA het plan voor pediatrisch onderzoek heeft goedgekeurd en voor welke klassen de EMA akkoord is gegaan met ontheffing van de noodzaak tot het doen van pediatrisch onderzoek in de periode 2007 tot maart 2012. Wij zijn tevens nagegaan of de Verordening leidt tot de ontwikkeling van geneesmiddelklassen waarvoor een (niet voldane) behoefte bestaat voor pediatrisch onderzoek ('*(unmet) pediatric need*') of tot de ontwikkeling van klassen met de hoogste farmaceutische uitgaven. Bovendien hebben vijftig Nederlandse artsen die met kinderen werken aangegeven of zij vinden dat de geneesmiddelen (die onder

de Verordening voor kinderen worden ontwikkeld) daadwerkelijk nodig zijn. In de periode 2007 tot maart 2012 werd ongeveer tweederde van de geneesmiddelen door de EMA goedgekeurd voor pediatrisch onderzoek; uitstel van de start of afronding van maatregelen in het onderzoeksplan tot na de autorisatie van het geneesmiddel voor volwassenen werd door de EMA gehonoreerd voor 83% van deze geneesmiddelen. Geneesmiddelklassen en therapeutische subgroepen met een hoge noodzaak tot pediatrisch onderzoek op de EMA *Needs Lists*, zoals geneesmiddelen met een effect op het centrale zenuwstelsel, werden of weinig frequent goedgekeurd voor pediatrisch onderzoek of regelmatig ontheven van deze noodzaak. Bovendien werden geneesmiddelen die regelmatig worden voorgeschreven aan kinderen of adolescenten (PHARMO Database Network, 2005-2011), maar niet altijd goed beschikbaar zijn (en hierdoor een '*unmet pediatric need*' hebben) relatief zelden onder de Verordening goedgekeurd voor pediatrisch onderzoek. Geneesmiddelklassen met het laagste aantal geneesmiddelen waarvoor pediatrisch onderzoek was goedgekeurd waren klassen met de laagste farmaceutische uitgaven. Tenslotte waren de vijftig Nederlandse artsen er niet van overtuigd dat de geneesmiddelen waarvoor pediatrisch onderzoek was goedgekeurd noodzakelijk waren voor de klinische praktijk.

Wij concluderen dat de belangrijkste maatregel van de Verordening niet noodzakelijkerwijs leidt tot toename van geneesmiddelonderzoek bij kinderen van geneesmiddelklassen waarvoor een (*unmet) pediatric need* bestaat. De output van de Verordening komt overeen met farmaceutische uitgaven, waarschijnlijk door de '*adult-driven*' aanpak. Door het hoge aantal geneesmiddelen waarvoor uitstel van onderzoek door de EMA werd gehonoreerd blijft de registratie van geneesmiddelen met een effect op het centrale zenuwstelsel bij kinderen en adolescenten nog altijd achter bij die bij volwassenen. Wij stellen daarom een aantal belangrijke verbeteringen voor die nodig zijn in de implementatie van de regelgeving om ervoor te zorgen dat de Verordening leidt tot geneesmiddelen die kinderen en adolescenten daadwerkelijk nodig hebben. Bijvoorbeeld, evaluatie door de EMA van een Pediatrische Onderzoeksplan (of verzoek om ontheffing van deze verplichting) dient te worden gebaseerd op de mogelijke pediatrische relevantie van het werkingsmechanisme of geneesmiddelentarget en er kunnen nieuwe stimuleringsmaatregelen worden overwogen voor *first-in-children* indicaties.

HOOFDSTUK 3: BIOMARKERS VAN ACUTE EFFECTEN VAN METHYLFENIDAAT BIJ KINDEREN EN ADOLESCENTEN MET ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

Methylfenidaat (MPH) is het meest frequent voorgeschreven geneesmiddel voor de behandeling van *attention-deficit/hyperactivity disorder* (ADHD) bij kinderen. Eerder gepubliceerde studies die het effect van *immediate-release* methylfenidaat (MPH-IR) onderzochten, hebben tot tegengestelde resultaten geleid, onder ander door verschillende bronnen van variabiliteit zoals het gebrek aan gestandaardiseerde biomarkers voor het meten van een geneesmiddeleffect.

De studie die wordt beschreven in hoofdstuk 3 was een systematische literatuur reviewstudie waarin sensitieve en bruikbare non-invasieve biomarkers werden geïdentificeerd voor het monitoren van effecten van MPH-IR bij kinderen en adolescenten met ADHD. In publicaties tot december 2009 werden 78 gerandomiseerde, placebo-gecontroleerde klinische studies gevonden die de effecten op het centrale zenuwstelsel onderzochten van een enkele dosis van MPH-IR bij kinderen of adolescenten met ADHD. Om tot een redelijke mate van standaardisering tussen studies en testen te komen, werden de uitkomstmaten geclusterd in groepen van gerelateerde testen of testvarianten ('clusters'). Door de progressieve bundeling van de resultaten in logische clusters kon een meer algemene beoordeling worden gedaan van het geneesmiddeleffect op groepen van vergelijkbare testen of functionele domeinen. Neurocognitieve clusters en individuele testen die in vijf of meer studies waren onderzocht werden geëvalueerd voor consistentie in de gemeten MPH effecten.

De resultaten van deze review toonden aan dat er veel verschillende biomarkers worden toegepast om de effecten van MPH-IR bij ADHD te evalueren. De volgende uitkomstmaten toonden een consistente respons op

een therapeutische dosis van MPH in verschillende studies en verschillende cohorten: *Continuous Performance Test*, *Go/no-go Task*, *Visual Evoked Potentials* en verschillende observatieschalen (inclusief *Following Rules Observations*, *Oppositional Behavior Observations*, *On-Task Behavior Observations* en *Impulsivity Behavior Observations*). Het effect van MPH was het best te detecteren in testen en observaties gerelateerd aan motorische controle, volgehouden aandacht, gedeelde aandacht en impulsiviteit (controle van inhibitie); dit betekent dat MPH acute effecten heeft op de drie kernsymptomen van ADHD (aandachtstekort, hyperactiviteit en impulsiviteit) bij kinderen met ADHD die een effect ondervinden van MPH.

Wij stellen voor dat de potentiële biomarkers die in deze review zijn geïdentificeerd mogelijk kunnen bijdragen aan de identificatie van *responders* versus *non-responders* na een testdosis met MPH. Omdat het niet mogelijk was om dosis-effect relaties vast te stellen, dienen deze testen en clusters verder te worden onderzocht op dosis-respons relaties, inclusief de effectgrootte, en op klinisch relevante veranderingen. Idealiter worden in deze studies naast dosis-effect relaties ook concentratie-effect relaties onderzocht op verschillende tijdspunten na toediening van MPH, zodat het effectprofiel van MPH bij kinderen en adolescenten met ADHD kan worden vastgesteld.

HOOFDSTUK 4: BEPALING VAN METHYLFENIDAAT IN PLASMA EN SPEEKSEL DOOR LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

Door het monitoren van MPH-concentraties kan een bijdrage worden geleverd aan het bepalen of de afwezigheid van een effect of de aanwezigheid van onverwachte bijwerkingen gerelateerd zijn aan farmacokinetische of farmacodynamische factoren. Therapeutisch monitoren van MPH-behandeling bij volwassenen wordt meestal gedaan door het meten van MPH-concentraties in plasma. Bij kinderen is het afnemen van bloedmonsters echter ongewenst. Speeksel is mogelijk een alternatieve matrix voor het monitoren van MPH-concentraties; echter, er zijn verschillende potentieel complicerende factoren naar voren gekomen in eerdere studies. Complicerende factoren waren onder andere indicaties van orale contaminatie in de eerste speekselsamples na inname van MPH en een aanzienlijke – nog onverklaarde – variatie in de S/P ratio na toediening van zowel de tablet als de capsule.

Het verkrijgen van een betrouwbare S/P ratio is essentieel voor het behalen van het volledige potentiaal van het gebruik van speekselmonsters voor het monitoren van MPH-concentraties. Om deze reden hebben wij een analytische methode ontwikkeld voor het nauwkeurig kwantificeren van MPH in zowel plasma als speeksel. In dit hoofdstuk presenteren wij de validatie van een *liquid chromatography-tandem mass* spectrometriemethode waarbij gebruik wordt gemaakt van een *hydrophilic interaction liquid chromatography column* (HILIC). Eiwitten werden geprecipiteerd in een 100 µl monster met 750 µl acetonitrile/methanol 84/16 (v/v) dat d_9-methylfenidaat bevatte als interne standaard. Er werden standaardcurves geplot over een MPH-concentratiebereik van 0.5 – 100.0 µg/L. De totale analysetijd was 45 seconden. De accuraatheid bevond zich in het bereik van 98-108% en de *within-* en *between-run* onnauwkeurigheid was minder dan 7.0%. De matrixeffecten waren groter voor plasma dan voor speeksel met 46% and 8% ionisatiesuppressie. De matrixeffecten werden adequaat gecompenseerd door het gebruik van gedeutereerd MPH als interne standaard. MPH degradeerde significant in plasma en speeksel op kamertemperatuur en bij een temperatuur van 5°C. Stabiliteitsexperimenten toonden aan dat de monsters direct na afname bij een temperatuur van -20°C of lager moeten worden bewaard en dat de monsters direct na ontdooien moeten worden verwerkt. De monsters waren voor tenminste 4 weken stabiel bij een temperatuur van -20°C. De methode werd met succes toegepast voor de bepaling van MPH-concentraties in plasma en speeksel van een volwassen gezonde vrijwilliger.

Wij concluderen dat door het gebruik van eiwitprecipitatie en *hydrophilic interaction liquid chromatography* gecombineerd met *tandem mass* spectrometrie, deze methode tot een snelle, betrouwbare en correcte kwantificatie van MPH leidt in zowel plasma als speeksel.

HOOFDSTUK 5: POPULATIEFARMACOKINETISCH MODEL VAN TWEE TOEDIENINGSVORMEN VAN METHYLFENIDAAT IN PLASMA EN SPEEKSEL VAN GEZONDE VRIJWILLIGERS

In dit hoofdstuk wordt een studie beschreven waarin een eerste aanzet is gedaan om de bronnen van variabiliteit in MPH plasma- en speekselconcentraties te kwantificeren en om de relatie tussen MPH-concentratie in speeksel en MPH-concentratie in plasma te beschrijven door middel van een populatiefarmacokinetisch model.

In deze gerandomiseerde, open-label studie werden 10 mg MPH-IR (tablet) en 18 mg *osmotic release oral system* MPH (MPH-OROS, capsule) gekruist toegediend aan 12 volwassen gezonde vrijwilligers (zes mannen en zes vrouwen). Zowel bloed- als speekselmonsters werden voorafgaand aan en regelmatig na toediening van MPH afgenomen tot 6 (MPH-IR) of 11 (MPH-OROS) uur na toediening. De farmacokinetische analyses zijn uitgevoerd met het populatiefarmacokinetieksoftwarepakket NONMEM (*nonlinear mixed-effect modeling*).

Farmacokinetiek van MPH in plasma werd het best beschreven door een 1-compartimentenmodel met eerste-orde-absorptie (met aparte compartimenten voor MPH-IR en MPH-OROS) en eerste-orde-eliminatie. De geschatte klaring was 6.0 liter/uur en het distributievolume was 7.5 liter. De terminale halfwaardetijd was 0.9 uur. Interindividuele variabilititeit werd geïdentificeerd voor klaring, distributievolume en de absorptiesnelheidsconstante van MPH-OROS. De S/P ratio was 2.44 vanaf 2.5 uur na toediening. Interindividuele variabiliteit werd geïdentificeerd voor de S/P ratio.

Wij verwachten dat dit PK model met juiste allometrische schalingstechnieken bij kinderen gebruikt kan worden om het concentratie/tijdsprofiel in plasma te voorspellen op basis van gemeten MPH-concentraties in speeksel. De voorspellende prestatie van het model bij kinderen met ADHD behoeft nog verder onderzoek.

HOOFDSTUK 6: FARMACOKINETIEK EN EFFECTEN VAN CAFEÏNE OP PARAMETERS VAN HET CENTRALE AND AUTONOME ZENUWSTELSEL BIJ ADOLESCENTEN

Kinderen en adolescenten gebruiken regelmatig cafeïne als psychostimulans. Ondanks het veelvuldige gebruik van cafeïne door adolescenten, is er relatief weinig onderzoek gedaan naar de fysiologische en gedragsmatige effecten van cafeïne in deze leeftijdsgroep. Data uit dierstudies suggereren dat de effecten van cafeïne bij volwassenen niet eenvoudig geëxtrapoleerd kunnen worden naar adolescenten.

Om deze reden hebben wij in deze studie het effect van cafeïne op parameters van het centrale en autonome zenuwstelsel onderzocht bij gezonde adolescenten na inname van een cafeïnehoudende drank (met een lage dosis); de resultaten werden vergeleken met data verkregen na inname van een cafeinevrije drank. Cafeïneconcentraties werden gemeten in speekselmonsters. In een aparte studie bij gezonde volwassenen hebben wij de mate van orale contaminatie met cafeïne bepaald na inname van cafeïnehoudende drank versus een cafeïnehoudende capsule (200 mg). Speeksel- en plasmamonsters werden tegelijkertijd afgenomen om de S/P ratio van de cafeïneconcentraties te bepalen. Een populatiefarmacokinetisch model werd opgesteld gebaseerd op data uit deze kinetische studie om zo de plasmaconcentraties bij de eerdere studie bij adolescenten te schatten; dit model kon vervolgens worden gebruikt om een farmacokinetiek-farmacodynamiek (PK/PD) model op te stellen.

Cafeïne had bij adolescenten een significant effect op testparameters gerelateerd aan aandacht en visuomotore coördinatie (*adaptive tracking task*) en alertheid (*saccadic peak velocity*). Na inname van cafeïne werd bovendien een toename in de *error* rate van de *attention switch task* waargenomen. Plasmaconcentraties van cafeïne werden bij volwassenen het best beschreven door een 2-compartimentenmodel met een dosisdepot, eerste-orde-absorptie- en eerste-orde-eliminatiekinetiek. Het plasmamodel identificeerde een dosis van 90 mg in de cafeïnehoudende drank. Vetvrije massa (*lean body mass*) -afhankelijke variabiliteit werd geïdentificeerd voor het volume van het

centrale compartiment. Het farmacokinetische model werd uitgebreid naar een populatiemodel waarin cafeïneconcentraties in speeksel bij volwassenen als een fractie (0,68) van de plasmaconcentraties werden beschreven vanaf 1 uur na toediening; in andere woorden, de S/P ratio was vanaf 1 uur na toediening 0,68. Tot 1 uur na toedining kon de speekselconcentratie niet als een fractie worden beschreven; om deze reden waren effecten van cafeïne die binnen 1 uur na toediening optraden niet geschikt voor inclusie in een PK/PD model.

Wij concluderen dat 90 mg cafeïne bij gezonde adolescenten een significant effect heeft op parameters gerelateerd aan alertheid en reactiesnelheid, ondanks de relatief lage dosis en het verwachte plafondeffect bij deze gezonde en alerte populatie. Verder onderzoek is nodig om te achterhalen of deze effecten bij adolescenten groter zijn dan bij volwassenen.

HOOFDSTUK 7: VERGELIJKING TUSSEN GEZONDE ADOLESCENTEN EN VOLWASSENEN VAN DE FARMACOKINETIEK EN DE EFFECTEN OP OBJECTIEVE EN SUBJECTIEVE BIOMARKERS VAN ALCOHOL

Ons begrip van leeftijdsafhankelijke verschillen in de farmacokinetiek en de farmacodynamiek van geneesmiddelen met een effect op het centrale zenuwstelsel is bijna volledig gebaseerd op diermodellen. In dit hoofdstuk wordt een studie beschreven waarin wij een PK/PD model hebben opgesteld om de objectieve en subjectieve effecten van alcohol tussen adolescenten en volwassenen te vergelijken. Het acute effect van inname van een sociaal-geaccepteerde dosis alcohol (2 standaardeenheden) werd bepaald bij 16-18 jarige adolescenten. Alcoholconcentraties werden non-invasief gemeten door middel van eind-expiratoire ademmonsters. Vervolgens is een PK/PD model opgesteld aan de hand van data van deze studie en data van eerder uitgevoerde alcoholstudies (met de *clamping* methode) bij volwassenen. Dit model werd gebruikt om de farmacokinetiek en effecten op een objectieve en op een subjectieve biomarker te karakteriseren en mogelijke bronnen van variabiliteit, zoals leeftijd, te onderzoeken.

Alcoholkinetiek in plasma werd het beste beschreven door een 2-compartimentenmodel met eerste-orde-absorptie en Michaelis-Menten-eliminatie. Interindividuele variabiliteit werd geïdentificeerd voor verschillende kinetische parameters. Er was sprake van vetvrije massa (*lean body weight*)-afhankelijke variabiliteit in het volume van het perifere compartiment en de maximale eliminatie, gewichtsafhankelijke variabiliteit in het volume van het centrale compartiment en lengte- en leeftijdsafhankelijke variabiliteit in intercompartimentele klaring.

Gebaseerd op een verkennende meta-analyse van alle relevante alcoholdata werden *smooth pursuit performance* en VAS *Alertness* geselecteerd als biomarkers voor het opstellen van het PK/PD model. De relatie tussen alcoholconcentratie en de effecten van alcohol op *baseline smooth pursuit performance* and VAS *Alertness score* werd het best beschreven als een dosis-afhankelijke relatie, zonder indicaties van vertraging of tolerantie. Hogere *baseline smooth pursuit performance* was gecorreleerd met een grotere absolute afname in uitvoering. Voor *smooth pursuit performance* en VAS *Alertnes* werden geen covariaten geïdentificeerd voor de relatie tussen alcoholconcentratie en -effect.

De covariaten die in ons PK-model werden geïdentificeerd kunnen (in-) direct gerelateerd zijn aan verschillen tussen adolescenten en volwassenen, aangezien aanzienlijke leeftijdsafhankelijke en rijpingsafhankelijke verschillen in lichaamssamenstelling optreden gedurende de adolescentie. VAS *Alertness* en *smooth pursuit eye movements* vertegenwoordigen niet noodzakelijkerwijs alle alcoholgerelateerde effecten op het centrale zenuwstelsel en wij kunnen niet uitsluiten dat de gevoeligheid voor andere alcoholgerelateerde farmacodynamische effecten met de leeftijd verandert. Helaas waren andere gevoelige functionele biomarkers voor het effect van alcohol minder geschikt voor het opstellen van een PK/PD model, doordat het kleine effect bij adolescenten en het hoge aantal *non-responders* ons belette om de data bij adolescenten te kwantificeren en een leeftijdseffect te onderzoeken. Om deze reden moet nog worden vastgesteld of de gevoeligheid voor andere alcoholgerelateerde effecten verandert met de leeftijd.

HOOFDSTUK 8: FARMACOKINETIEK VAN MELATONINE MINITABLETTEN MET VERLENGDE AFGIFTE BIJ KINDEREN MET EEN AUTISME SPECTRUM STOORNIS EN EEN SLAAPSTOORNIS

Melatonine is één van de middelen met een effect op het centrale zenuwstelsel waarvoor de EMA een pediatrische therapeutische noodzaak tot onderzoek heeft geïdentificeerd. Dit omvat de noodzaak tot het ontwikkelen van een leeftijdsgeschikte toedieningsvorm met verlengde afgifte en de noodzaak tot het verzamelen van data over de farmacokinetiek, werkzaamheid en veiligheid bij kinderen met autisme en een slaapstoornis.

De studie beschreven in hoofdstuk 8 maakt onderdeel uit van een Pediatrisch Ontwikkelplan (PIP) onder de Europese pediatrische Verordening. Deze studie was een gekruiste studie met oplopende doseringen van Circadin (1 mg minitabletten met een diameter van 3 mm), een toedieningsvorm met verlengde afgifte. In deze studie zijn het farmacokinetische profiel, de veiligheid en aanvaardbaarheid van Circadin geëvalueerd bij 16 kinderen en adolescenten met autisme spectrum stoornis en een slaapstoornis. Gebaseerd op de dosisrange in een toekomstige studie waarin de werkzaamheid zal worden onderzocht (die ook onderdeel is van de PIP) werden doseringen van 2 en 10 mg getest. Om afname van monsters gedurende de nacht zoveel mogelijk te beperken, namen de kinderen Circadin 's ochtends in. De eerste studiedag werd voorafgegaan door een 24-uur baseline meting van melatonine. Speekselmonsters werden non-invasief verkregen door passief kwijlen en de melatonineconcentratie in de monsters werd gemeten. Urinemonsters werden verzameld voor de bepaling van de metaboliet 6-sulfatoxymelatonine (6-SMT). Gedurende de studie werd gemonitord voor het optreden van bijwerkingen en sedatieve effecten werden geëvalueerd door middel van afname van de *Observer's Assessment of Alertness/Sedation* (OAA/S) schaal tot 10 uur na toediening. De farmacokinetische analyses zijn uitgevoerd met NONMEM.

Alle 16 deelnemers (12 man, 4 vrouw; leeftijd: 7-15 jaar) hadden de klinische diagnose autisme spectrum stoornis (gebaseerd op DSM-IV-TR criteria). Alle gerapporteerde bijwerkingen waren bekende bijwerkingen van melatonine. De minitabletten waren veilig (geen van de kinderen verslikte zich) en werden door de kinderen geaccepteerd. De piekconcentratie trad binnen 2 uur op en melatonineconcentraties bleven gedurende enkele uren hierna verhoogd. Blootstelling aan Circadin was dosis-lineair en de klaring (1.000 L/u) was vergelijkbaar tussen de dosisgroepen. De terminale halfwaardetijd was vergelijkbaar tussen de doseringen. De gemiddelde hoeveelheid urine 6-SMT tijdens de baseline periode was 4,2 µg (gedurende de eerste 12 uren overdag) en 13,5 µg (gedurende de volgende 12 uren 's nachts). Na toediening van 2 mg Circadin was de gemiddelde hoeveelheid urine 6-SMT 989,5 µg (gedurende de eerste 12 uren overdag) en 95,3 µg (gedurende de volgende 12 uren 's nachts). De hoogste sedatiescores (OAA/S) traden op tussen 2 en 3 uur na toediening van 2 mg Circadin en tussen 2 en 6 uur na toediening van 10 mg Circadin. De deelnemers en hun verzorgers waren in het algemeen positief over de belasting en duur van de studie. Bijna alle deelnemers (69%) en verzorgers (88%) zouden (toestemming voor) deelname aan een vergelijkbare studie weer overwegen.

Wij concluderen dat deze studie de korte termijn veiligheid en aanvaardbaarheid aantoont en het farmacokinetische profiel van verlengde afgifte demonstreert van Circadin minitabletten bij 16 schoolgaande kinderen en adolescenten met autisme spectrum stoornis.

HOOFDSTUK 9: FARMACOKINETIEK EN FARMACODYNAMIEK VAN EEN NIEUWE HOOG-GECONCENTREERDE INTRANASALE TOEDIENINSGVORM VAN MIDAZOLAM VOOR MATIGE SEDATIE

Vanwege de snelle werking en het snelle herstel is midazolam het geneesmiddel van eerste keus voor het verkrijgen van matige sedatie (voorheen ook wel *conscious sedation* genoemd) en de management van epileptische aanvallen. Nasale toediening van midazolam is een non-invasief alternatief voor intraveneuze toediening. Eerder ontwikkelde nasale toedieningsvormen van midazolam zijn weinig succesvol gebleken door het gebrek aan solvens waarin midazolam in therapeutische doseringen kan worden opgelost zonder dat er schade ontstaat aan de nasale mucosa.

In de studie die wordt beschreven in hoofdstuk 9 is de farmacokinetiek van twee doseringen van een nieuwe hoog-geconcentreerde waterige intranasale toedieningsvorm van midazolam (Nazolam) gekarakteriseerd. In dit vier-weg gekruiste, dubbelblinde, dubbel-*dummy*, gerandomiseerde, placebo-gecontroleerde onderzoek ontvingen 16 volwassen deelnemers 2,5 mg Nazolam, 5,0 mg Nazolam, 2,5 mg intraveneuze midazolam of placebo op verschillende studiedagen. De farmacokinetiek van midazolam en α-hydroxy-midazolam werden gekarakteriseerd en gerelateerd aan uitkomstvariabelen voor sedatie (*saccadic peak velocity*, *Bond and Lader Visual Analogue Scale for sedation*, *simple reaction time task* en de *Observer's Assessment of Alertness/Sedation*). De aanvang en duur van het farmacologische effect werden geëvalueerd en vergeleken met intraveneus toegediende midazolam door middel van de biomarker *saccadic peak velocity* (SPV), aangezien er een relatie is beschreven tussen afname in de SPV en klinische werking. Nasale tolerantie werd geëvalueerd op basis van spontane rapportage door deelnemers en onderzoek van de neus.

De biologische beschikbaarheid van Nazolam was 75%. Maximale plasmaconcentraties van 31 ng/ml (CV, 42.3%) werden bereikt na 11 minuten (2,5 mg Nazolam) en van 66 ng/ml (CV, 31.5%) na 14 minuten (5,0 mg Nazolam). De aanvang van sedatie (gebaseerd op een -2SD verandering in SPV) trad 1 minuut na toediening van 2,5 mg intraveneuze midazolam, 7 minuten na 2,5 mg Nazolam, en 4 minuten na 5 mg Nazolam op. Duur van de sedatie was 85 minuten voor 2,5 mg intraveneuze midazolam, 47 minuten voor 2,5 mg Nazolam en 106 minuten voor 5,0 mg Nazolam. Klinisch relevante levels van sedatie (gemeten door middel van de OAA/S) werden binnen minuten na toediening bereikt. De eenmalige toediening werd bovendien goed verdragen, was veilig en leidde niet tot beschadiging van de nasale mucosa.

Wij concluderen dat deze studie de nasale tolerantie, korte-termijn veiligheid en werkzaamheid van deze nieuwe formulering aantoont bij 16 volwassen gezonde deelnemers. Matige sedatie kan in hetzelfde tijdbestek worden behaald indien de voorbereidingstijd die nodig is voor het verkrijgen van een intraveneuze toegangsweg overwogen wordt. Deze non-invasieve toedieningsvorm heeft potentieel belangrijke voordelen voor matige sedatie en epilepsie en heeft een mogelijke toepassing bij kinderen.

HOOFDSTUK 10: ALGEMENE DISCUSSIE

Geneesmiddelen met een effect op het centrale zenuwstelsel worden onder de Verordening relatief weinig frequent onderzocht, ondanks de noodzaak tot pediatrisch onderzoek en de aanhoudende toename in het gebruik van deze geneesmiddelen bij kinderen en adolescenten. Afgaande op onze data is het onwaarschijnlijk dat de Verordening een positieve uitwerking zal hebben op de toegang van kinderen en adolescenten tot nieuwe geneesmiddelen met een effect op het centrale zenuwstelsel (aangezien er weinig van dit soort nieuwe geneesmiddelen voor volwassenen wordt ontwikkeld) of op de vertraging in pediatrische registratie van deze geneesmiddelen, zoals beschreven in de Introductie van dit proefschrift. Aangezien de meerderheid van de ontwikkelplannen voor geneesmiddelen met een effect op het centrale zenuwstelsel uitgestelde onderdelen bevat, is het belangrijk om *clinical trial strategies* tijdig te herzien.

In de Introductie van dit proefschrift stelden wij voor dat wanneer de variabilititeit in de S/P ratio gekwantificeerd of geminimaliseerd kan worden, het meten van geneesmiddelconcentraties in speeksel een zinvol alternatief kan zijn voor het meten van geneesmiddelconcentraties in plasma voor meer geneesmiddelen dan tot op heden is aangenomen. De studies beschreven in hoofdstuk 5 en 6 zijn een aanzet om deze bronnen van variabiliteit te onderzoeken en te kwantificeren door middel van een populatiefarmacokinetische model. Wij toonden aan dat de relatie tussen plasma- en speekselconcentraties voor MPH stabiel is vanaf 2,5 uur na toediening en voor cafeïne vanaf 1 uur na toediening. Voorafgaand aan deze tijdspunten was de relatie niet lineair; helaas waren onze pogingen om te corrigeren voor factoren die mogelijk bijdragen aan de variabiliteit in de S/Pratio niet succesvol. Voor geneesmiddelen met een korte T_{max} (zoals MPH en cafeïne) is het aantal speekselmonsters dat verzameld kan worden om de absorptiefase vast te stellen beperkt, waardoor de bepaling van de mate van contaminatie, de S/Pratio en bronnen van

variabiliteit in de eerste periode na toediening wordt belemmerd. Voor dit type geneesmiddelen zijn alleen effecten die relatief laat optreden geschikt voor het opstellen van een PK/PD model gebaseerd op gemeten speekselconcentraties of geschatte plasmaconcentraties. Speekselafname is echter mogelijk nog steeds een nuttige optie voor dit type onderzoek, omdat het farmacologische effect op het centrale zenuwstelsel van de meeste geneesmiddelen vertraagd is ten opzichte van veranderingen in de plasmaconcentratie. Om deze reden ondersteunen onze resultaten het verder onderzoeken van het gebruik van speeksel als een non-invasieve methode voor het profileren van geneesmiddelen met een effect op het centrale zenuwstelsel.

De non-invasieve testen die opgenomen zijn in de NeuroCart testbatterij zijn voldoende gevoelig om effecten van lage doseringen van cafeïne en alcohol bij gezonde adolescenten te detecteren (zoals wij aantoonden in hoofdstuk 5 en 6). Na toediening van cafeïne werden significante effecten gezien op parameters gerelateerd aan alertheid (*saccadic peak velocity*) en reactietijd (*adaptive tracking*), ondanks de relatief lage dosering en het verwachtte plafondeffect in dit cohort van gezonde en alerte adolescenten. Omdat gerapporteerd is dat cafeïne vergelijkbare effecten heeft op het gedrag (zoals motorische activatie en *arousal*) als klassieke psychostimulantia zoals cocaïne en amfetamine, zijn deze testen mogelijk van toegevoegde waarde in pediatrische studies met andere stimulantia. Een lage dosering alcohol leidde tot significante veranderingen in *smooth pursuit eye movements*, VAS *Alertness score*, VAS *alcohol effect score*, *body sway*, systolische bloeddruk en hartfrequentie. In diermodellen zijn ontwikkelingsgerelateerde veranderingen beschreven in de farmacologische gevoeligheid van $GABA_A$-receptor-gemedieerde *currents* in reactie op verschillende geneesmiddelen. Om deze reden kunnen deze taken wellicht gebruikt worden om leeftijdsafhankelijke veranderingen te onderzoeken in de effecten van geneesmiddelen die aangrijpen op GABA. De meerderheid van de adolescente deelnemers verdroeg de testen en de testduur goed.

In de Introductie van dit proefschrift brachten wij een aantal punten naar voren ten aanzien van neuropsychofarmacologie. Ten eerste is het belangrijk om de verschillen in neuropsychopathologie en –farmacologie tussen kinderen en volwassenen te erkennen. Bovendien bestaat bij onderzoekers de behoefte aan gevalideerde methoden die geschikt zijn voor de evaluatie van de werkzaamheid en veiligheid van geneesmiddelen met een effect op het centrale zenuwstelsel. Tenslotte moet er nadruk worden gelegd op het onderzoek naar toedieningsvormen. Wij stellen voor dat het profileren van geneesmiddelen met een effect op het centrale zenuwstelsel het potentiaal heeft om een bijdrage te leveren aan al deze punten. Ten eerste kunnen leeftijdsafhankelijke effecten worden geëvalueerd door het geneesmiddelprofiel bij kinderen of adolescenten te vergelijken met een (reeds eerder vastgesteld) profiel bij volwassenen, zoals gedemonstreerd is door de studie beschreven in hoofdstuk 7. Ten tweede laten verschillende geneesmiddelklassen een uniek effectprofiel zien op de testen uit de NeuroCart testbatterij dat overeenkomt met het werkingsmechanisme van het middel. Wij hebben eerder voorgesteld dat deze 'vingerafdrukken' gebruikt kunnen worden om te differentiëren tussen een stimulerende en sedatieve geneesmiddeleigenschap, bijvoorbeeld door het effect op *saccadic peak velocity* te onderzoeken. Zoals reeds genoemd, zijn de testen in de NeuroCart voldoende gevoelig om de effecten van een lage dosering cafeïne of alcohol bij adolescenten te detecteren. Functionele biomarkers kunnen worden gebruikt om te bepalen of een specifiek geneesmiddel bij kinderen of adolescenten in een bepaalde dosering tot een farmacologisch effect leidt. Deze biomarkers kunen ook worden gebruikt om (on)gewenste sedatieve of stimulerende eigenschappen van geneesmiddelen bij kinderen en adolescenten te onderzoeken. Ten derde kan het non-invasief monitoren van de farmacokinetiek en farmacodynamiek onderzoek naar toedieningsvormen bij kinderen en adolescenten vereenvoudigen, zoals geïllustreerd door de studies die zijn beschreven in hoofdstuk 8 en 9. De '*proof-of-pharmacology*' strategie die is beschreven in hoofdstuk 9 kan wellicht ook worden gevolgd bij pediatrische studies waarin een leeftijdsgeschikte toedieningsvorm wordt onderzocht voor een geneesmiddel waarvoor reeds veel data bestaan over de werkzaamheid en veiligheid.

Verschillende stappen moeten worden ondernomen in aanvulling op verdere verfijning van de methoden die in dit proefschrift zijn beschreven. Allereerst moet non-invasieve monitoring van geneesmiddelen worden geëvalueerd bij jongere kinderen en bij kinderen met andere neuropsychiatrische aandoeningen. Een tweede belangrijke stap, met name gezien het feit dat de meest neurologische en psychatrische aandoeningen een chronisch of recidiverend karakter hebben, is het bepalen of vroege behandeling leidt tot acute verbetering van symptoomgerelateerde functies van het centrale zenuwstelsel en verbeterde lange-termijn uitkomsten. Tenslotte, vanwege de noodzaak om de effecten van anesthetica en analgetica te onderzoeken bij kinderen en adolescenten, dient er meer ervaring te worden op gedaan op het gebied van pediatrisch pijnonderzoek.

Conclusie

De klinische studies beschreven in dit proefschrift tonen aan dat het non-invasief vaststellen van een geneesmiddelprofiel van geneesmiddelen die het centrale zenuwstelsel stimuleren of onderdrukken uitvoerbaar is bij gezonde adolescenten en bij kinderen en adolescenten met een autisme spectrum stoornis. Deze aanpak draagt bij aan de evaluatie van leeftijdsafhankelijke verschillen in farmacokinetiek en farmacodynamiek en de vergelijking van effectprofielen van verschillende toedieningsvormen van hetzelfde geneesmiddel. Deze aanpak vergemakkelijkt mogelijk bovendien de uitvoering van studies die onderdeel zijn van een Pediatrisch Ontwikkelplan onder de Verordening; gezien de noodzaak voor pediatrisch onderzoek op het gebied van geneesmiddelen met een effect op het centrale zenuwstelsel zou dit een belangrijke stap zijn. De ideale pediatrische studie dient zo opgesteld te zijn dat deze in de thuissituatie kan plaatsvinden. De S/P ratio moet worden bepaald in studies bij volwassenen met nieuwe geneesmiddelen waarvan de concentraties mogelijk in speekselmonsters kunnen worden gemeten. Omdat aanzienlijke variabiliteit in de farmacokinetiek is beschreven voor verschillende geneesmiddelen voor neuropsychiatrische aandoeningen, dienen gevoelige non-invasieve of minimaal-invasieve farmacodynamische metingen longitudinaal te worden verricht. Pediatrische studies dienen bovendien zo te zijn opgesteld dat een vergelijking met studies bij volwassenen mogelijk is. Tenslotte, om onze kennis over de aanvaardbaarheid van verschillende meetmethoden te vergroten is het nodig (indien mogelijk) deelnemers te vragen een vragenlijst in te vullen om zo informatie te verzamelen over de redenen van deelname en over de ervaren onderzoeksbelasting.

CURRICULUM VITAE

Lenneke Schrier was born on August 9, 1979 in Den Helder (The Netherlands). Between 1999 and 2004, during her study Medicine, she was a member of the International Federation of Medical Students' Associations-The Netherlands, an organization that aims to improve Global Health and the personal development of future health care professionals. During this period, she initiated several projects within the field of health and human rights. In 2001 and 2002 she presided over the national board, and from 2002 until 2004, she was a member of the Supervisory Board of the national board. In 2004 and 2005, she worked as a pre-doctoral visiting research fellow at the lab of Dr. Jeffrey Baron at the National Institute of Child Health and Human Development (NICHD, NIH, Bethesda, United States). The Section headed by Dr. Baron investigates the cellular and molecular mechanisms governing childhood growth and development. During this period, she studied the depletion of resting zone chondrocytes during growth plate senescence in an animal model, which led to a publication in an international journal. Between 2005 and 2007, she worked (temporarily) as a pharmacovigilance assessor at the Dutch Medicines Evaluation Board (The Hague, The Netherlands). During this work, she noticed the lack of data on efficacy and safety of drugs in children, and this observation made her want to contribute to knowledge in this area through scientific research and education. In 2007, she was involved in the World Health Organization (WHO) Project 'Make Medicines Child Size', which aims to lead to increased access to effective, safe and affordable medicines for children. Within this project, she assisted in the development of the Essential Medicines List for Neonates (WHO Department Policy and Standards). For this work, the Leiden University Fund awarded her with the Janneke Fruin-Helb Beurs, an award aimed at the encouragement of excellent students that have received a Leiden University International Fund grant for research internships abroad. This award enabled her to participate in the European Course on Evaluation of Medicinal Products in Children in 2008. The extracurricular activities described above were done whilst studying Medicine. After obtaining her medical degree (*with honours*) in 2008, she started working as a pediatric resident at the Reinier de Graaf Ziekenhuis in Delft (The Netherlands). Early 2009, she started her PhD program at the Centre for Human Drug Research (CHDR) – in collaboration with the Willem-Alexander Children's Hospital (WAKZ) – in Leiden (The Netherlands), under supervision of Prof. dr. Adam Cohen and Prof. dr. Joop van Gerven (CHDR), and Dr. Rám Sukhai (WAKZ), a position she held until 2012. During this period, monthly input was provided by the Pediatric Pharmacology Network, consisting of several pediatricians, including Prof. dr. Jan Maarten Wit (WAKZ), Alfred van Meurs (Juliana Children's Hospital, The Hague, The Netherlands) and Rob Pereira (Pediatric Department Maasstad Ziekenhuis, Rotterdam, The Netherlands). Most of the research described in this thesis was performed within this period. Whilst working at the CHDR, she was trained as a clinical pharmacologist; she obtained her certification in 2014. Her interest in non-invasive monitoring of drug (side) effects on the central nervous system in children and adolescents led to a 1-year training in Radiology at the Erasmus Medical Center (Rotterdam, The Netherlands) under supervision of Dr. Winnifred van Lankeren in 2013. Since June 2014, she is in training as a pediatric resident under supervision of Dr. Wouter Kollen at the WAKZ.

LIST OF PUBLICATIONS

J Burggraaf, I Kamerling, P Gordon, L Schier, ML de Kam, A Kales, R Bendiksen, A Langers, G Torheim, M Warren, H Morreau, G Dalsgaard, A Healy, J Hardwick. Detection of colonic neoplasia in vivo in humans using an intravenously administered fluorescent peptide targeted against c-Met and fluorescent colonoscopy. *Nature Methods (In Press)*

L Schrier, ML de Kam, R McKinnon, A Che Bakri, W Oostdijk, TC Sas, LA Menke, BJ Otten, SM de Muinck Keizer- Schrama, B Kristrom, C Ankarberg-Lindgren, J Burggraaf, K Albertsson-Wikland, JM Wit. Comparison of Body Surface Area versus Weight-Based Growth Hormone dosing for girls with Turner Syndrome. *Horm Res Paediatr.* 2014;81:319-30.

A Secilir, L Schrier, Y Bijleveld, J Toersche, S Jorjani, J Burggraaf, J van Gerven, R Mathot. Determination of methylphenidate in plasma and saliva by liquid chromotography/tandem mass spectroscpy. J *Chromatogr B Analyt Technol Biomed Life Sci.* 2013;923-924:22-8.

M Moerland, AJ Kales, L Schrier, MG van Dongen, D Bradnock, J Burggraaf. Evaluation of the EndoPAT as a tool to assess endothelial function. *Int J Vasc Med.* 2012;2012:904141.

R Marino, A Hegde, KM Barnes, L Schrier, JA Emons, O Nilsson, J Baron. Catch-up growth after hypothyroidism is caused by delayed growth plate senescence. *Endocrinology* 2008;149(4):1820-8.

L Schrier, SP Ferns, KM Barnes, JA Emons, EI Newman, O Nilsson, J Baron. Depletion of resting zone chondrocytes during growth plate senescence. *J Endocrinol.* 2006;189(1):27-36.

O Nilsson, RD Mitchum Jr, L Schrier, SP Ferns, KM Barnes, JF Troedle, J Baron. Growth plate senescense is associated with loss of DNA methylation. *J Endocrinol.* 2005;186(1):241-9.