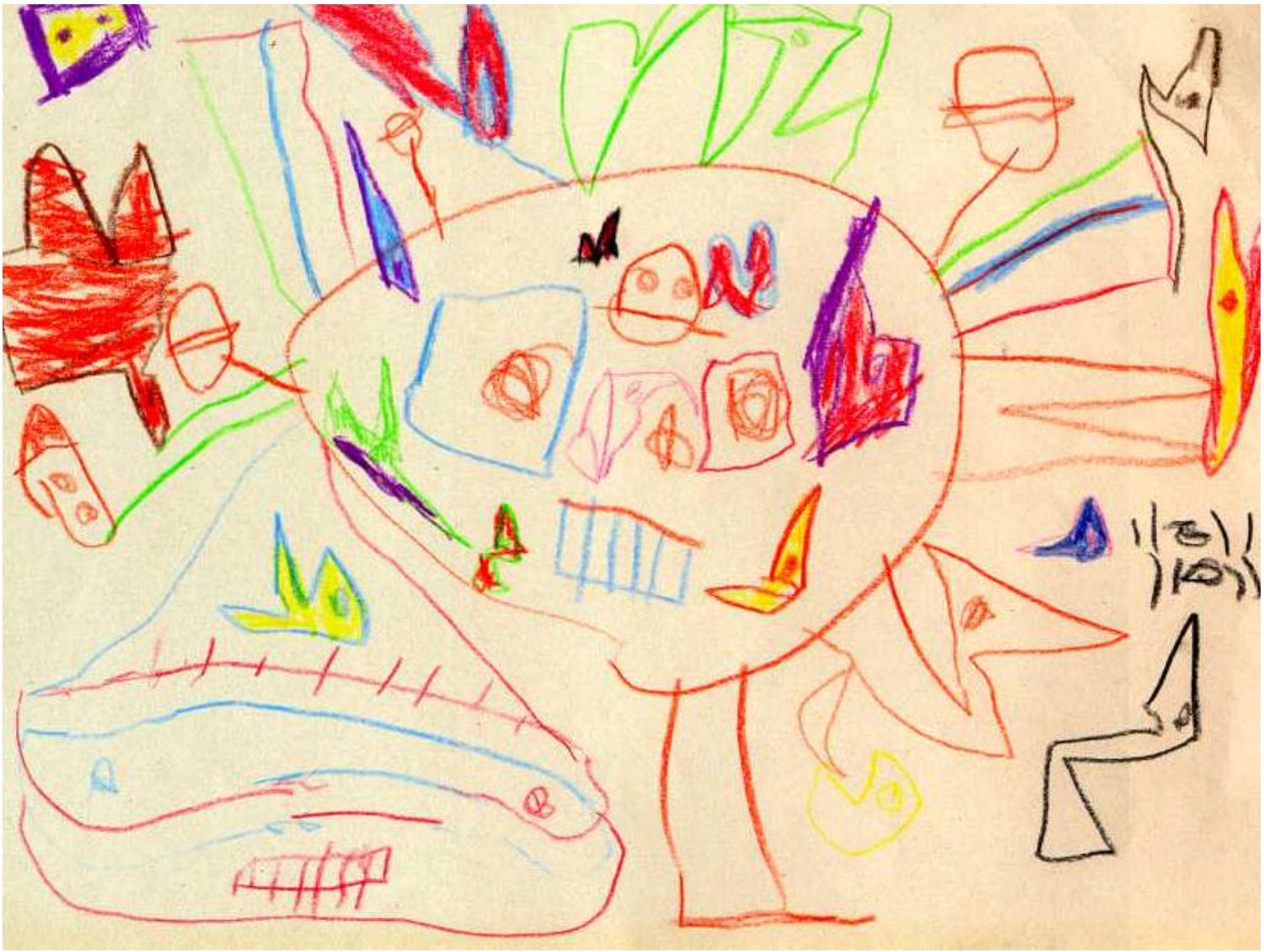

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# Modelling repeated measurements in clinical pharmacology; from individual to population and back

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# Modelling repeated measurements in clinical pharmacology; from individual to population and back

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| Promotor | Prof. Dr. A.F. Cohen |
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Cover illustration: Maxim Schoemaker

# Stellingen

behorend bij het proefschrift ›Modelling repeated measurements in clinical pharmacology; from individual to population and back‹

1. *Onmogelijk* is een kwestie van perspectief.
   Dit proefschrift
2. *Empirical Bayes estimates* maken het delen van informatie tussen individuen mogelijk en *mixed effect modelling* toegankelijk.
   Dit proefschrift
3. Het vertalen van een *indocyanine groen* concentratie profiel naar het onderliggende klaringsprofiel is met slechts een beperkt aantal vooronderstellingen mogelijk.
   Dit proefschrift
4. Het schatten van de $EC_{50}$ parameter uit het $E_{max}$ model moet bij *in-vivo PK/PD modelling* vervangen worden door het schatten van de $S_0$ parameter, gegeven door $E_{max}/EC_{50}$.
   Dit proefschrift
5. Toegepaste statistiek is één deel computatie en negen delen communicatie.
6. Bij de keuze van een statistische methode moet de meest efficiënte overdracht van informatie het basisprincipe zijn en de *bloody obvious test* het uitgangspunt.
7. De aanwezigheid van significante autocorrelatie bij analyse van herhaalde metingen duidt op het onvoldoende modelleren van het structurele model voor het individu; het is daarom een duidelijke aanwijzing voor verder inhoudelijk onderzoek.
8. Het concept van *individual bioequivalence* biedt een statistische oplossing voor een niet bestaand probleem.
9. Gezien de aard en incidentie van sportblessures, moet het suggereren van een causaal verband tussen sport en gezondheid met de grootst mogelijke argwaan bekeken worden.
10. De vraag aan zorgende mannen of het vandaag jouw beurt is om op te passen, geeft blijk van een merkwaardige kijk op vaderschap; oppassen doe je op andermans kinderen.
11. Mannen en vrouwen met kinderen die aangeven dat een eerlijke verdeling tussen arbeid en zorg wenselijk, maar in *hun* situatie niet mogelijk is, zijn gewoon niet bereid de consequenties te dragen.
12. Je bedenken is een teken van kracht.
    Christopher Lloyd

Rik Schoemaker, Leiden, 4 maart 1999

# Theses

1. *Impossible* is a matter of perspective.
   This thesis

2. *Empirical Bayes estimates* enable sharing of information between individuals and make *mixed effect modelling* accessible.
   This thesis

3. Translation of an *indocyanine green* concentration profile into the underlying clearance profile is possible with only a limited number of assumptions.
   This thesis

4. Estimation of the $EC_{50}$ parameter in the $E_{max}$ model should, in the context of *in-vivo PK/PD modelling*, be replaced by estimation of the $S_0$ parameter, defined by $E_{max}/EC_{50}$.
   This thesis

5. Applied statistics is one part computation and nine parts communication.

6 The most efficient transfer of information must be the guiding principle in the choice of a statistical method and the *bloody obvious test* must be the point of departure.

7. The presence of significant autocorrelation in the analysis of repeated measurements indicates insufficient modelling of the structural model for the individual; it is therefore a clear indication for additional conceptual research.

8. The concept of *individual bioequivalence* offers a statistical solution to a non-existing problem.

9. The suggestion of a causal link between sports and health must -in view of the nature and incidence of sports injuries- be viewed with great suspicion.

10. Approaching a man who takes care of his children with the question ›is it your turn to baby-sit?‹ exposes a curious view of fatherhood; one only baby-sits other people‹s children.

11. Men and women with children who indicate that a fair division between work and care is desirable but impossible in *their* situation, are simply not prepared to bear the consequences.

12. Changing your mind is a sign of strength.
    Christopher Lloyd

Footnote: It is tradition at most Dutch universities to accompany the PhD thesis by a number of theses (stellingen). These theses are statements which the candidate considers to be true. A number of them pertain to the thesis itself, several should be related to the academic field of the candidate and the rest are of a more personal nature. They are an integral part of the thesis and may therefore be questioned during the defence. As they are generally posed in Dutch and will therefore elude the foreign reader, I‹ve attempted a translation. Regrettably, some of the finer points may have been lost in the process (notably for number 10 and perhaps number 11), for which I apologise.

Rik Schoemaker, Leiden, March 4 1999

*En wederom is het kwaad als kauwgom onder het tafelblad der gerechtigheid*

*Superworm Jim*

*voor mijn moeder*

*zonder wie ik niet zou zijn*

*voor mijn vader*

*zonder wie ik nooit was begonnen*

*voor Marieke*

*zonder wie ik al veel eerder klaar zou zijn geweest,*

*maar dat wat telt, had gemist*

# Table of contents

# Preface

Clinical pharmacology experiments offer the unique opportunity to carefully investigate pharmacology in human subjects under strictly controlled experimental conditions. A key aspect of this process is taking a series of measurements at consecutive time points to document the effect of a treatment over time on the individual subject. The analysis of these measurements is the subject of this thesis.

If interest lies in describing and quantifying the outcome of a study, then often model-independent summary measures suffice. If the results are meant for extrapolation, for generalisation or for understanding or gaining insights into underlying mechanisms, then mathematical models must be used that allow the data to be interpreted in the context of a larger framework of existing or postulated knowledge.

The main body of this thesis is devoted to finding solutions to practical problems in analysis of repeated measures data encountered during work at the Centre for Human Drug Research. Several aspects are central to these solutions. First, they aim to extract the maximum amount of information from the data. Second, they deal with identifiability, i.e. whether identifying certain aspects of the data or underlying process is possible, like treatment response profiles, parameter values or competing models. Third, they provide solutions to the problem of dealing with missing information on part of the individual subjects.

Maximal information extraction, solving identifiability issues and dealing with missing information generally require the combining of information over all subjects and mathematical models that capture important aspects of the underlying process. The statistical technique of mixed effect modelling is well suited for this purpose and is applied in most chapters. Mixed effect modelling capitalises on the notion that all subjects are different but have much in common at the same time. Instead of independently estimating parameters for each subject, average parameters and inter-individual variability estimates are generated. This allows the sharing of information across subjects where missing information of some is supplemented by information available from others.

Although simple summary measures may be preferable for presentation of basic trial outcomes, this thesis shows that models are useful for increasing the understanding of the data. The techniques to analyse the repeated measurement data properly in these situations are investigated, explained and made practically accessible.

## Overview of the thesis

Chapter 1 provides an overview of methods for the analysis of continuous repeated measurement data.

Chapter 2 deals with the analysis of experiments where each subject is both measured repeatedly over time and on different occasions (associated with different treatments). If data are missing, the standard repeated measures analysis of variance approach collapses. Several solutions are examined like filling in estimates for the missing data. Theory and simulations indicate that the best choice is the use of linear mixed effect models with a simple 'small sample' correction for significance testing.

Chapter 3 provides an introduction into nonlinear mixed effect models and their use in data-rich situations. A main strength in using these models is the ability for individuals to borrow information from other subjects. This leads to more stable and coherent parameter estimates for the individual and for the entire group. Several examples are presented to illustrate their usefulness in a clinical pharmacology setting.

Chapter 4 deals with the modelling of the pharmacokinetic profile of drugs that are rapidly and extensively cleared by the liver. By administering a constant rate infusion of marker compound, a continuous assessment of the hepatic clearance may be obtained which can be related to changes in liver blood flow. Both semi-parametric models and parametric models requiring systems of differential equations are presented.

Chapter 5 provides a solution to estimating model parameters for the $E_{max}$ concentration-effect model when maximal effects are not attained. Deviations from a linear concentration-effect relationship are commonly encountered but estimates of $EC_{50}$ and $E_{max}$ are very unreliable if a maximum is not reached. By estimating a different parameter (called $S_0$), the situation becomes manageable, allowing comparison of the potency of different drugs that only exhibit partial $E_{max}$ profiles or comparison of increasing doses of the same drug.

Chapter 6 provides an application of model discrimination to *in vitro* pharmacology data. It is shown that competing but unrelated concentration-effect models may be formally compared through the use of a super-model. This requires nonlinear mixed effect modelling to adequately estimate the model parameters.

Finally, the summary and conclusions summarises the thesis and shows that maximal extraction of information, identifiability and dealing with missing information that are the motivating forces for the presented solutions, may be achieved using appropriate modelling techniques.

Chapter 1

# Analysis of continuous repeated measurements in clinical pharmacology

**Summary**

This chapter provides an overview of analysis methods for studies with repeated measurements on the same individual. Only analysis of continuous outcomes is discussed. If the data allow it, simple summary measures are preferable to more complex methods that analyse the entire profile. In the cases where these simple methods fail, statistical techniques like mixed effect modelling are available that allow the combining of information of varying quality and quantity. Experiments with missing measurements or missing information on part of the subjects may also be successfully analysed using these methods.

## Introduction

Clinical pharmacology experiments often entail the repeated assessment of subjects under different conditions. The analysis of series of measurements in such subjects on consecutive time points is the subject of this thesis. Many procedures can be applied to these data and this chapter aims to provide a global overview.

The emphasis of the techniques discussed is on analysis of continuous (as opposed to categorical, dichotomous or count) data. Although the effects of drugs are ultimately aimed at inducing or preventing the occurrence of clinical events, these may fall outside the realm of clinical pharmacology. Study of these events often requires large scale trials, often with few measurements per subject. A wealth of statistical methods (e.g. survival analysis, logistic regression) is available for the analysis of these data [1,2] but they are beyond the scope of this thesis.

The analysis of repeated measurements can be divided into two main categories: the reduction of the stream of data into summary measures to be subsequently analysed, and the analysis of the entire data set as a whole.

The most straightforward way of analysing a sequence of measurements is by reducing the sequence to a small number of characteristics [3,4]. Reduction of a profile into summary measures may be done without specific underlying models (model-independent methods) or by estimating the parameters of mathematical models that can be thought to explain aspects of the studied process.

Profile-reduction may become problematic if measurements are missing or if individuals provide varying amounts of information. In these cases it may be preferable to model a structure to the data and analyse the data set as a whole, without prior reduction. By combining information over all the individuals and analysing them collectively, sensible parameter estimates may be obtained, that would otherwise remain unavailable. The statistical class of mixed effects models is very useful for this purpose and will be described.

## Summary measures

The repeated assessment in time of subjects under different conditions or treatments introduces a complex structure in the data; within-subject measurements for a particular treatment are often correlated and if subjects receive different treatments an additional level of dependency is created. If individual time points are compared between treatments (using Student-s t-tests for instance) then this dependency structure is ignored and test results will not be independent. Moreover, treatment-induced changes in the measured time profile may be best described by

changes in certain characteristics of this profile rather than by changes at individual time points.

These problems may be solved by reducing the original sequence into a small number of summary measures. These summary measures abolish the repeated nature of the data, solving the problem of the correlated measurements, and allow description of the most important aspects of the profile. For these reasons, the use of summary measures has been advocated by for instance Matthews *et al* [4].

Model-independent summary measures are most useful at summarising the data, and provide a compact and comprehensible description of study outcomes. Additional information may be extracted from the measurements if a relevant mathematical model can be employed. The estimated model-parameters may allow generalisation and extrapolation of study results under certain conditions. The models can also provide a valuable tool for increasing the understanding of the underlying process.

*Model-independent summary measures*

Matthews *et al* [4] classify treatment responses as either of the peak or growth type. The peak type shows a clear increase followed by a decrease or *vice versa* while the growth type is characterised by a steady decrease or increase.

For treatment profiles with a clearly defined peak or trough, several useful summary measures may be employed. Maximal effects ($E_{max}$ or $C_{max}$ for concentrations) and the associated time ($T_{max}$) can be simply read from the data. Average response may be quantified by a mean or, if the data points are not equidistant, by an area under the curve (AUC). If this AUC is divided by the time span over which it was calculated, a weighted average response results.

For data with a clear response, an alternative useful measure may the time of onset of effect and the duration of effect. Onset of effect must be arbitrarily defined for instance as a set percentage increase from initial values or a predetermined shift from baseline. The time point associated with this shift may be obtained either by interpolation or by taking the first measurement for which it occurs. If the data are variable then additional criteria (for instance related to a minimal duration of effect) may be imposed to ensure that the onset of effect is real and not determined by measurement error.

For data that change in a constant direction over time, an estimate of the rate of change may be the most useful summary measure. Linear regression may be used to calculate this rate of change if the increase or decrease is linear over time. If linearity is not assured then sometimes a data transformation can be used. One could contemplate the use of polynomials if linearity cannot be achieved, but the resulting polynomial coefficients rarely have intrinsic meaning and will only be useful for an alternative description of the profile.

*Model-dependent summary measures*

If a mathematical model can be constructed that is consistent with the data then additional information may be extracted from the data. Examples are the deterministic models describing the fate of drugs in the body (compartmental pharmacokinetic models) and the models describing the relationship between drug concentrations and effects (pharmacokinetic/pharmacodynamic models).

Based on physiology, biology or more often empirical experience, mathematical models may be constructed describing concentrations or effect measurements. Pharmacokinetic models are and have been very successful although the basic ideas behind them like transfer into, between and out of compartments with immediate mixing, can only be approximations of actual physiology [5].

The delay in time called hysteresis that is often observed between plasma concentrations and corresponding effects may be dealt with in various ways. One empirical approach is to assume that concentrations in a compartment other than the site of sampling are more closely related to the effects, an Aeffect compartment@ may be implemented to account for the hysteresis [6]. In practice, such an effect compartment may not actually be related to a physiological compartment. It is therefore often called a hypothetical compartment and serves then as a computational tool.

A different class of models called indirect response models may account for a delay in effect by assuming that the drug influences the rate of formation or elimination of a substance linked to the effect [7]. Based on knowledge about the mode of action or the physiology of the system, these indirect response models may in some cases provide a more realistic description.

Both the indirect response models and the effect compartment models are simplifications of reality. If the models are used mainly as descriptors of the data without compelling arguments to the applicability of either one in a specific setting, their predicted behaviour can be distinguished in experiments by their response to varying input regimes. The effect compartment model predicts that the time of maximal concentration ($T_{max}$) is not influenced by the administered dose, while the indirect response model predicts a different $T_{max}$ for different doses [8].

Both classes of models have greatly increased the potential for describing the relationship between administered dose, resulting concentration and subsequent effect. It has been proposed that the use of these PK/PD models in drug development may substantially increase efficiency and will result in more valuable information in the drug development process [9].

Parameter estimation in nonlinear regression is an iterative computational procedure [10] where success may depend on the correct choice of starting values, the adequacy of the model, and informativeness of the data set. The choice of the mathematical entry of parameters in the model may also influences the estimation process. The same model (curve) may be estimated with different parameters; the basic one-compartment model can be parameterised using clearance and volume, but elimination half-life or elimination rate constant can also be estimated directly, combined with estimation of either clearance or volume. Rewriting the model with different parameters may result in much faster convergence and more stable estimates [11]. Unfortunately, the most computationally desirable parameters may not be meaningful at all in explaining the physiology, and therefore choice of parameterisation is rarely dictated by statistical considerations alone. An example of a stable and meaningful reparameterisation of the classic $E_{max}$ concentration-effect model is provided in chapter 5 of this thesis.

In the most common form of nonlinear regression, the dependent measurements like concentration can be written as a direct (explicit) function of the independent variables like time. The standard compartmental (sums of exponentials) pharmacokinetic models are common examples. Alternatively, the rate of change of a variable may be modelled as a function of this variable itself using differential equations. These differential equations are at the basis of all pharmacokinetic compartmental models where the rate of concentration change in a compartment is a function of the concentration itself and the rate of drug entry into the compartment. For special situations explicit solutions may exist, but if this is not so then the model-parameters can be estimated using the differential equations.

The physiologic indirect response models illustrate the requirement for parameter estimation using the differential equations [9]. In these models, the rate of formation or elimination of a substance (e.g. clotting factors) is influenced by another variable (e.g. warfarin concentration) and no explicit solutions exist. Chapter 4 of this thesis provides another example where the rate of elimination of the marker compound sorbitol is modelled as a function of changes in hepatic blood flow.

After estimating the parameters for each individual, these estimates may be combined and

compared between treatments. This will produce adequate results if each individual provides estimates of comparable accuracy. However, if estimates are not based on (roughly) the same number of measurements for each individual, or if the response for one individual is much more variable than for another, some form of weighting of the different estimates is appropriate [12]. This weighting of the relative contribution can also be obtained by the mixed effect models described in the next section.

## Mixed effect models

When individuals provide sufficient information of comparable quality across subjects, it is often preferable to calculate summary measures of the treatment response and subsequently analyse these measures between treatments using standard statistical methodology. However, when crucial measurements are missing, or when individual treatment responses provide insufficient information to obtain adequate individual parameter estimates, the data set will need to be analysed as a whole. In this way, information may be shared across subjects taking the relative contribution of each individual into account.

The statistical class of mixed effect models makes this sharing and combining of information possible. The models are called 'mixed' because they describe the data using a mixture of fixed and random effects. Fixed effects are under the control of the investigator like the time of measurement and the treatment administered, while random effects describe the variability in the measurements within or between subjects.

The power of the mixed effect models comes from the fact that differences in parameters between subjects are modelled using distributions for these parameters. The task of estimating individual parameters for each subject is replaced by estimating a single average and inter-individual variability estimate for each parameter.

### *Linear mixed effect models*

Data are often presented as curves of average effect over time. These curves will generally provide a good (initial) description of the data if the shape of the individual profiles is comparable for all subjects. If comparison of the entire profile between treatments is wanted, then linear mixed effect models provide the appropriate statistical methodology.

The models are called linear not because they make use of straight lines but because the models describe response as a linear combination of the levels of the factors within a design. Average response for a treatment at a particular time point is given by the sum of the average treatment response (over all time points), the average response at that time point (over all treatments) and the deviation for that particular treatment-time point combination. The analysis can answer three questions: 1) are the curves parallel?, 2) is one curve on average above the others?, and 3) does the average profile (irrespective of treatment) change over time?

If there are no missing data, then repeated measures analysis of variance (ANOVA) techniques may be used [3,13]. If there are missing data then several solutions may be entertained. Missing measurements may be replaced by reasonable estimates or the entire data set may be modelled. Chapter 2 of this thesis describes the various alternatives and investigates their applicability. The most viable approach is by using a linear mixed effect model and estimating the parameters using maximum likelihood.

If maximum likelihood is used then a wide range of missing measurements may be dealt with. Not only the completely random missing measurement can be incorporated but also more serious patterns of missing data. On theoretical grounds, the analysis is valid if the probability for a measurement of being missing is not determined by the missing measurement itself. This probability may, however, be influenced by preceding measurements. This means that if during treatment a subject drops out because of lack of relief by the treatment, his data can (and should) still be incorporated in the analysis. This situation could not be adequately dealt with using a summary measure like the AUC because it is undefined if measurements are missing at the end of the profile. Simply taking the average of available data will not be adequate either. The remaining subjects are likely to judge their condition more favourably. Otherwise, they would have taken escape medication as well.

Linear mixed effect models are not only flexible regarding missing measurements, but also in the choice of covariance structures that describe the correlation between the different time points and treatments. In practice, different covariance structures rarely lead to large differences in point estimates of the fixed effects, but statistical significance of these effects may be genuinely affected [3]. Random effects models can be extended into the realm of generalised linear mixed effect models [3]. These may be used to describe binary data, like presence or absence of symptoms after various challenge conditions, or counted responses like the number of events observed in a particular time span.

The price for the increased flexibility over the repeated measures ANOVA approach is the loss of exact and explicit solutions. The significance statements for mixed effects models rely on large sample arguments meaning they assume that an infinite number of subjects were studied. This can mean poor approximations in practice while the quality of the approximation cannot be easily established. Chapter 2 of this thesis provides a correction to the large sample approximations and presents simulation results that show that the correction works well for the design studied.

*Random coefficient linear regression*

Random coefficient linear regression models are a useful subclass of the linear mixed effect models. The essence of these models is that a common linear regression model is applied to all subjects, but coefficients (slopes and intercepts) are allowed to vary between subjects. These coefficients are the random effect, and only mean and (inter-individual) variance of the coefficients are estimated within the model. These population parameters may be used to test differences between treatments or between groups.

Random coefficient linear regression may be contrasted with the two-stage approach where derived variables are first estimated for each subject (individual regression coefficients) that are subsequently analysed using standard techniques. The difference between random coefficient regression and the two-stage approach is that each subject may supply a different amount of information because of a variable number of data points or data points in more or less pivotal positions. Random coefficient linear regression can allow for this while the two-stage approach generally assumes all estimates to be equally precise.

*Nonlinear mixed effect models*

Conceptually, it is a small step from random coefficient linear regression to nonlinear mixed effect models. The same principles apply, only the model for the individual is nonlinear in its parameters. The main and motivating advantage is the gathering of strength. Small amounts of information for each subject may be combined to provide a clear picture of model parameters on a population level.

Nonlinear mixed effect models were initially developed in the field of population analysis where small amounts of routinely collected kinetic data are gathered for many subjects to investigate the kinetic behaviour of drugs in the population actually taking the drugs. Additionally, explanatory covariates are sought to explain part of the inter-individual variability making dose adjustments possible [14].

The use of nonlinear mixed effect models is not restricted to these sparse data situations. In clinical pharmacology research settings, one may encounter data rich situations with many measurements per subject, but still have insufficient information to obtain adequate parameter estimates. Combining of information over subjects in these cases will result in better parameter estimates. Chapter 3 of this thesis deals with these situations.

The transfer of information is not necessarily only from individual to population. The population information may be used to improve the estimates of individuals with insufficient information. This can be accomplished by using empirical Bayes estimates. These individual estimates are called Bayes estimates because they are conditional on the population information. They are called empirical because the prior information is not obtained from an external source but from the data itself. The transfer of information is therefore from individual to population and back.

Nonlinear mixed effect models can be approached through a variety of statistical and computational techniques [15]. An extensive bibliography of methods and applications is provided by Yuh *et al* [16]. The method that has been around longest and has been implemented in the most flexible software program to date, is NONMEM [17,18].

There are two main analytical methods within the NONMEM program and both use a linear approximation to the nonlinear model to make computation feasible. As with ordinary nonlinear regression, computations are iterative, but the two methods differ in the way that the different subjects are accounted for. For the first-order method, all subjects have the same estimate for the parameters during a single iterative evaluation step. This numerical approximation may work if individual subjects supply only limited information but may lead to serious errors in data-rich situations. This flaw is avoided in the first-order conditional estimation method. It is based on the ideas presented by Lindstrom and Bates [19] and is implemented by generating individual empirical Bayes estimates during each iteration. This method, although of far greater numerical complexity and therefore requiring more extensive (computer) time, is advocated in data-rich situations and with highly nonlinear models (NONMEM users guide VII [18]).

Another useful application of nonlinear mixed effects modelling is the ability to discriminate between alternative models. With single-subject nonlinear regression, a model comparison would have to be done for each individual fit and the outcome subsequently somehow combined over subjects to allow a general statement of superiority of one of the models. This combining of test results is not trivial and may lead to contradictory results; for some subjects a significant improvement will be found while others may not show this. This problem is solved by estimating a single set of population parameters for each model using (nonlinear) mixed effect modelling.

A prerequisite for model discrimination is that models are nested, meaning that one model can be seen as a restricted version of another model, obtained for instance by fixing certain parameters. Classical likelihood ratio tests may be used to investigate whether the more complex model results in a significant increase in fit [10,20]. This for instance allows the formal testing of whether a two-compartment model provides a significant improvement over a one-compartment model, whether an $E_{max}$ model performs better than a linear model, or if inclusion of a hypothetical effect compartment is merited. In cases where the two models are not nested, formal testing is not supported by theory unless a 'super model' can be found for which either model is a specific case. Chapter 6 of this thesis provides such an example.

Although significance testing is possible using nonlinear mixed effect models, several uncertain factors remain. Significance tests rely on large sample justifications and this usually means that test results are only approximate. They may even be far off, and the only way currently known to examine whether the test results are valid in a specific situation, is to investigate them using a bootstrapping procedure [21]. Bootstrapping is implemented by repeatedly sampling and analysing subsets of the original data set. The distribution of the bootstrapped parameters provides an indication of the underlying parameter distribution. This, however, requires a large number of computer runs (perhaps more than 1000) which, at the current state of computing, is often unfeasible as a standard approach.

An additional problem in significance testing is generated by the large number of parameters that may be required for characterisation of the different treatments. For instance, testing whether the kinetics of a drug changes under different conditions may require different parameters for each of these conditions. This means that if several treatments are compared simultaneously, a prohibitively large number of parameters need to be estimated. This can pose great numerical difficulties, even if the data would allow adequate estimation of all the relevant parameters. For these reasons, the main use of nonlinear mixed effect modelling in data-rich situations may be in parameter estimation and not in significance testing.

## Conclusions

If summary measures can adequately capture the aspects of the response that are of interest, they are preferable to the use of the complex statistical techniques needed to analyse the entire data set as a whole. Presentation of results is much simplified and will not be clouded by unease with complex statistical methodology on the part of the reader.

However, if crucial information is missing for some of the subjects, or subjects provide information with varying degrees of accuracy, mixed effect modelling may allow adequate estimation of the relevant response parameters. Naturally, the more complex approach will require more explanation, but if this allows the extraction of information that would otherwise remain hidden, it may well be worth the effort.

The choice between model-independent and model-dependent approaches are closely related to the rationale for the study. If the aim is to describe, or to test simple clearly specified prior hypotheses, model-independent measures are generally adequate and have the advantage of ease of interpretation.

If the data can be placed in a larger conceptual framework, mathematical models may be available or be developed to describe aspects of the underlying process. Estimation of these model parameters may aid in increasing the knowledge about the process. If the models prove consistent over different situations, they may be used to generalise and sometimes extrapolate the results of the study.

Finally, models may increase in complexity during model-development. Nonlinear mixed effect modelling provides the methodology to compare these different models on a population level and to test whether the increase in complexity is substantiated by the data.

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Chapter 2

# Repeated measures for two within-subject factors; analysis and missing data solutions

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**Summary**

The statistical analysis of the Repeated Measures design with two factors within and no factors between subjects which is popular in clinical pharmacology, is discussed. Use of Restricted Maximum Likelihood (REML) methodology is compared to an Imputation procedure in small sample situations with missing data and is illustrated by simulations. While Imputation leads to undesirable results which are not easily corrected, REML estimation in which test statistics are compared to an F-distribution provides an elegant tool for the analysis of these designs.

## Introduction; design

In pharmacokinetic and pharmacodynamic research and many other fields of applied science, one is frequently interested in comparing the time course of an outcome variable related to two or more treatments, as the effects on blood pressure of different drugs or different dosages of the same drug. This time course can be investigated either by randomising the patients to one of the possible treatments or by administering all treatments to the same subject. The adequacy of the crossover design (all treatments in one subject) has been questioned [1], but most of the criticism may be mainly based on studies involving patients. Carry-over effects can be prominent when insufficient time is available between treatments or when the drug under investigation induces relatively long lasting changes (cure being an extreme example). The fact that the patients' health can vary considerably over time compounded by seasonal effects may complicate matters by causing complex period effects.

In clinical pharmacology however, short-lasting experiments are often performed in healthy volunteers. Treatments will be adequately spaced and will induce only transient and often relatively small effects. Period effects are minimised by keeping subjects under strictly controlled circumstances. Period effects caused by increasing skill at performing certain tests are minimised by scheduling training sessions prior to the start of treatment. Any remaining sources of error are balanced over treatments by randomising treatment order according to Latin squares balanced for first order carry-over effects. This means that each treatment precedes every other treatment equally often [2].

If care is taken to remove these sources of error, the design has the advantage of providing both between and within subject measures of variability and of producing more powerful tests, as subjects serve as their own controls. An additional advantage is that maximum information is obtained from volunteers that may be hard to come by. For all these reasons, the design is popular in clinical pharmacology. For instance, 30 out of the 133 papers (23%) published in the 1991 issues of the *British Journal of Clinical Pharmacology* used this design.

## Analysis

While the design is used frequently, the statistical analysis is not so standard. Approaches vary from comparisons per time point (33% of the previously cited papers) to 'fixed-factor' analyses (17%) and specific Repeated Measures techniques (47%).

Most statisticians would agree that overall tests of significance are preferable to simple comparisons per time point. They allow general statements about effects, are more powerful in absence of interaction and provide a safeguard against too many spurious test results. Naturally, overall tests can be followed by comparisons per time point when significant treatment by time effects are present in order to provide an indication for the cause of the significant interaction. Since both factors are within-subject factors, these comparisons per time point are within-subject comparisons.

The 'fixed-factor' approach extends the classic Latin-square analysis of variance which has only one measurement associated with each treatment [2]. The classic Latin-square ANOVA has a subject effect, a treatment effect and a period effect due to the period in which the treatment is administered. The 'fixed-factor' approach adds an extra time effect and a treatment by time interaction in order to incorporate the successive measurements in time for each treatment. All factors are modelled as fixed factors. The design removes additive period effects and is a natural extension to the widely accepted analysis of cross-over designs [3]. However, the 'fixed-factor' approach imposes a very strict variance-covariance matrix on the within-subject measurements. It not only demands that all variances must be equal, but all covariances both within and between treatments must be equal as well. This means that there will have to be the same correlation between measurements hours apart (within treatments) as between measurements weeks apart (between treatments); this may be highly restrictive.

A completely unrestricted covariance matrix on the other hand, may be obtained using the multivariate approach to Repeated Measures treated by Cole and Grizzle [4]. While this procedure may seem preferable since it makes fewer assumptions, it has less power in situations where a more restricted covariance matrix is plausible. When the analysis is performed for a small number of subjects, Reinsel [5] has shown that the procedure may result in inaccurate estimates for the fixed effects. Finally, when the number of levels for a multivariately tested factor exceeds the number of subjects, significance tests are impossible because of singularity of the error matrix.

The univariate approach to Repeated Measures is based on the mixed model analysis of variance where the subject factor is assumed random [6]. This is the standard approach to Repeated Measures incorporated in software like SAS/STAT GLM [7], SPSS MANOVA [8] and BMDP4V [9] (along with the multivariate alternative) and is obtained by specifying the measurements as REPEATED, WSFACTORS and WITHIN FACTORS respectively. The restrictions on the covariance matrix are not as severe as in the 'fixed-factor' approach and the analysis is not hampered by the 'small sample' difficulties encountered in the multivariate approach.

The model can be described by the following equation:

$$X_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + (\pi)_k + (\alpha\pi)_{ik} + (\beta\pi)_{jk} + (\varepsilon)_{ijk}$$

Where $\mu$ = intercept (grand mean), $\alpha$ = treatment factor with i=1..t,
$\beta$ = time factor with j=1..u and $\pi$ = subject factor with k=1..s.
The treatment by time by subject interaction ($\alpha\beta\pi$) is indistinguishable from the residual error ($\varepsilon$) since there are no replications in the cells.
The factors ($\pi$), ($\alpha\pi$), ($\beta\pi$) and ($\varepsilon$) are random and mutually independent with:
$(\pi) \cong N(0,\sigma^2_{\pi})$, $(\alpha\pi) \cong N(0,\sigma^2_{\alpha\pi})$, $(\beta\pi) \cong N(0,\sigma^2_{\beta\pi})$ and $(\varepsilon) \cong N(0,\sigma^2_{\varepsilon})$.
The within-subjects variance-covariance matrix associated with this model
can be described in terms of a linear combination of these variance components. For a specific example with two treatment levels and two different time points this matrix amounts to:

$$\sigma^2_p * \begin{pmatrix} 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 \\ 1 & 1 & 1 & 1 \end{pmatrix} + \sigma^2_p * \begin{pmatrix} 1 & 1 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 0 & 0 & 1 & 1 \\ 0 & 0 & 1 & 1 \end{pmatrix} + \sigma^2_{ap} * \begin{pmatrix} 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 \\ 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 \end{pmatrix} + \sigma^2_e * \begin{pmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix}$$

This covariance matrix allows a different correlation between the same time points in different treatments than between the different time points in one treatment.
The Expected Mean Squares associated with this model are:

$E(MS\alpha) = \sigma^2_{\varepsilon} + u\sigma^2_{\alpha\pi} + u\sigma^2_{\alpha}$
$E(MS\beta) = \sigma^2_{\varepsilon} + t\sigma^2_{\beta\pi} + t\sigma^2_{\beta}$
$E(MS\alpha\beta) = \sigma^2_{\varepsilon} + s\sigma^2_{\alpha\beta}$
$E(MS\pi) = \sigma^2_{\varepsilon} + t\sigma^2_{\beta\pi} + u\sigma^2_{\alpha\pi} + tu\sigma^2_{\pi}$
$E(MS\alpha\pi) = \sigma^2_{\varepsilon} + u\sigma^2_{\alpha\pi}$
$E(MS\beta\pi) = \sigma^2_{\varepsilon} + t\sigma^2_{\beta\pi}$
$E(MS\varepsilon) = \sigma^2_{\varepsilon}$

These indicate that the $\alpha$ and $\beta$ main effects should be tested over their respective interactions with subjects, and the $\alpha\beta$ interaction over residual error. The deviation of the intercept from zero can be tested over the $MS\pi$. Corrections to these significance tests can be applied when the actual covariance matrix deviates from the specified 'doubly compound structure'. Both the conservative Greenhouse-Geiser epsilon [10] and the more liberal Huynh-Feldt epsilon [11] can be used to modify the degrees of freedom for the F-tests.

If one is especially concerned about the presence of additive period effects, these can be

removed by including design variables defining the period effect as covariates. This removes the variability attributable to the period effect from the 'treatment' error term and produces exactly the same results for treatment and period effects as a classic Latin-square analysis carried out on unweighted averages over the different time points in a treatment. Time and treatment by time effects are independent of the presumed period effect and are therefore unaffected by inclusion of this period effect in the model.

### Missing data

When some of the measurements are missing, standard analyses can not be applied and alternatives have to be found. Before any attempt at analysis can be made however, the mechanism causing the missing data must be considered. An excellent introduction into this matter is given by Laird [12] largely based on notions developed by Rubin [13].

The first distinction to be made is whether or not the missing data mechanism is ignorable from a likelihood based perspective. If the unobserved outcome itself causes the data to be missing, the mechanism is non-ignorable. This would be the case for instance, if measurements are discarded when above or below a specific value. Analysis of non-ignorable missing data can only be performed if the mechanism causing the missing data is explicitly modelled. This makes it a situation which is hard to approach with standard solutions and the rest of the paper will therefore only deal with ignorable missing data.

If the missing data mechanism is ignorable, this means that the likelihood does not have to be altered to cover the missing measurements.

The strictest demand on ignorable missing data, generally required by non-likelihood based analyses, is for the data to be 'missing completely at random' (MCAR). In this situation, the probability of a specific measurement being missing is not allowed to depend on observed variables. The accidentally spilled test-tube falls into this category.

If data are 'missing at random' (MAR), other variables are allowed to influence the probability of measurements being missing. It is therefore a weaker demand on ignorable missing data than MCAR. This may occur when certain treatment-time combinations have higher drop-outs, or when a treatment is discontinued *after* the outcome has reached critical levels. If one is certain that it is not the measurement itself which is behind the missing data mechanism, these frequently occurring situations can in theory be dealt with by likelihood-based analyses. However, they will rely heavily on the correct specification of the model.

**Missing data analysis**

There is no consensus on how data should be analysed when some of the measurements are missing. The problem is of course not new, and many solutions have been described.

The most simple but least desirable approach to a missing data situation is eliminating the entire subject who has missing data. With two factors within subjects this generally means deleting an unacceptable amount of information, while the data still have to be 'missing completely at random' (MCAR) for the resulting analysis to be valid.

Another solution is obtained by replacing the missing measurements by reasonable values and subsequently performing a complete data analysis. The classic choice for replacements, due in general to Yates [14], is the least squares estimate of the missing values. Little & Rubin [15] provide a comprehensive discussion. When there is only one error term, estimates can be found by using special formulas [16] or by iteratively estimating and imputing (filling in) values until the residual Sum of Squares is minimised [17]. If the number of missing values is subtracted from the degrees of freedom for the residual Sum of Squares, the correct Mean Square is obtained. Non-iterative solutions that give correct degrees of freedom can be obtained by Bartlett's ANCOVA method [18] expanded by Rubin [19] or by direct calculation of the least squares solution to the model using for instance SAS/STAT GLM [4] or SPSS MANOVA [5]. Even though the Hypothesis Mean Squares are overestimated [20] these methods are usually adequate provided the data are MCAR.

When there are multiple error terms as in the univariate Repeated Measures analysis, the 'least-squares' solution is problematic because it is unclear which error term or what combination of error terms should be minimised. An approximate solution could be conceived by initially taking all effects as fixed (including interactions with subjects), and subsequently using the Expected Mean Squares (obtained with for instance the SAS/STAT GLM RANDOM option [4]) for constructing synthetic F-values. However, the question remains which effect should incorporate the missing degrees of freedom in this approach. SPSS MANOVA [5] for instance randomly distributes these missing degrees of freedom over the various error terms (beginning with $MS_{\pi}$) while SAS/STAT GLM [4] removes them from the highest interaction term ($MS_{\alpha\beta\pi} = MS_{\varepsilon}$).

The most viable approach is probably through the use of Maximum Likelihood. Jennrich and Schluchter [21] have described a method for analysing Repeated Measures data by explicitly modelling the within-subject covariance matrix. Their methodology is implemented in the 5V module of BMDP [6]. A variety of covariance structures are pre-programmed including compound structure, first order autoregressive and general autoregressive structures for one within-subject factor. An unstructured matrix is available providing the multivariate Repeated Measures analysis. The 'doubly compound structure' required for two within-subject factors can be defined by the user with the general linear option as a linear combination of the four matrices associated with the four different variance parameters. A syntax description is provided in the Appendix.

A choice out of five minimising algorithms can be made, two of which provide Restricted

Maximum Likelihood (REML) estimates. REML estimates account for the loss in degrees of freedom from estimating the fixed effects and provide the same variance estimates as the corresponding ANOVA when data are complete [22]. Significance testing is performed using Wald statistics which are asymptotically distributed according to a chi-square distribution. A chi-square distributed variable with p degrees of freedom, when divided by p, follows an F-distribution with p and $\infty$ degrees of freedom. In all cases we have examined *with complete data* these Wald statistics (under REML estimation), when divided by their degrees of freedom, are identical to the F-statistics from the corresponding Repeated Measures ANOVA. Since Wald statistics are asymptotically distributed as a chi-square while many clinical pharmacology experiments are usually based on no more than 12 subjects, the discrepancy between for instance 11 and $\infty$ denominator degrees of freedom may lead to very liberal test results. In small sample situations, Berk [23] suggests comparing the divided Wald statistics to an F-distribution with degrees of freedom equal to the complete data Repeated Measures ANOVA situation, which may still be liberal but is certainly preferable to the 'infinite' number of subjects assumed for the asymptotic chi-square approach.

The new SAS/STAT procedure MIXED also provides ML and REML estimation in mixed effects models with structured covariance matrices [24,25] similar to BMDP5V. It provides 'F' values, suggests denominator degrees of freedom and allows the user to specify their own. However, the general linear covariance structure has not yet been implemented in the current version (Release 6.07) and therefore the procedure MIXED is not yet suitable for the univariate Repeated Measures approach with two factors within subjects.

## Simulation studies

A simulation study was performed to investigate the performance of the REML methodology and the adequacy of the proposed small sample adjustments. This was contrasted with the performance of an Imputation scheme (filling in for the missing values) followed by complete data analysis to examine whether this may be an acceptable alternative in practice.

The simulated data arise from a cross-over design in which six subjects receive two treatments and are measured on seven consecutive time points; 84 measurements in all. Two data sets with 500 replications each were generated differing in their covariance structure. For set I all covariances were set at zero. Set II used the scaled rounded average parameters obtained from the analysis of 20 real data sets: $\sigma^2_{\pi}$=25, $\sigma^2_{\alpha\pi}$ =5, $\sigma^2_{\beta\pi}$ =1, $\sigma^2_{\varepsilon}$ =10. From these data 0, 4, 8 and 16 measurements were randomly deleted corresponding to approximately 0% 5% 10% and 20% missing data. No fixed effects were incorporated so the simulation results give the distribution of the test-statistics for the three null-hypothesis of treatment, time and treatment by time effect.

For REML analysis BMDP5V was used with the 'REML' algorithm and with the user-defined covariance matrix for the univariate Repeated Measures approach (syntax in the Appendix). P-values were calculated by comparing the Wald statistics to the relevant chi-

square and F-distributions.

For imputation, missing values were replaced by the predicted values from the single-error term model obtained by performing ordinary least squares regression on the 'design matrix' with a subject, treatment, time and treatment by time effect only (see for instance Rawlings [26] or any other handbook treating ANOVA from a regression point of view). The resulting data sets were subsequently analysed using univariate Repeated Measures ANOVA.

The simulation results are summarised in Table I which gives the type I errors actually obtained while aiming at a level of 5%. The columns marked I and II designate the two data sets which differ in their covariance matrix. No systematic differences between these two situations can be detected.

Type I errors for the REML approach are clearly overestimated when the chi-square is used as a reference distribution. Dividing the Wald statistic by its degrees of freedom and comparing the result to an F-distribution with degrees of freedom equal to the complete data Repeated Measures ANOVA situation, results in type I errors much closer to the desired level.

Type I errors obtained with the Imputation approach rise substantially with increasing missing data fractions. Expected Mean Squares can be computed for this Imputation method and a derivation is provided in the Appendix. Unfortunately, these expectations not only depend on the missing data fraction (in which case a simple correction factor might be constructed) but also on the missing data pattern. The Mean Squares are contaminated with variance components which are absent in the complete data situation. Moreover, the numerator Mean Squares are too large while denominator Mean Squares are too small.

The data sets in the simulation have their missing data randomly scattered but a distinct missing data pattern may lead to even greater deviations of the Expected Mean Squares. Assume for instance that four subjects have their last four measurements missing for treatment 1.

**Table I.** Type I errors (500 samples) obtained for a nominal level of 5%
I=no covariances modelled; II='realistic' covariances

| | REML $\chi^2$ | | REML F | | Imputation | |
|---|---|---|---|---|---|---|
| Treatment | I | II | I | II | I | II |
| (df = 1,5) | | | | | | |
| 0% missing | 12.6 | 13.8 | 5.6 | 5.8 | 5.6 | 5.8 |
| 5% missing | 14.2 | 13.8 | 7.8 | 6.4 | 9.2 | 7.8 |
| 10% missing | 15.4 | 12.8 | 8.4 | 6.4 | 10.0 | 8.2 |
| 20% missing | 15.2 | 11.0 | 8.2 | 6.6 | 12.4 | 11.8 |
| Time | I | II | I | II | I | II |
| (df = 6,30) | | | | | | |
| 0% missing | 8.8 | 9.4 | 4.8 | 5.0 | 4.8 | 5.0 |
| 5% missing | 8.8 | 10.2 | 4.8 | 5.6 | 8.2 | 9.6 |
| 10% missing | 8.0 | 10.0 | 5.4 | 6.2 | 10.8 | 12.6 |
| 20% missing | 10.6 | 8.6 | 6.2 | 6.0 | 25.8 | 27.4 |
| Treatment X Time | I | II | I | II | I | II |
| (df = 6,30) | | | | | | |
| 0% missing | 6.8 | 6.6 | 4.0 | 3.4 | 4.0 | 3.4 |
| 5% missing | 7.4 | 7.2 | 4.4 | 3.8 | 7.2 | 7.2 |
| 10% missing | 8.8 | 7.4 | 5.6 | 4.2 | 11.6 | 10.8 |
| 20% missing | 9.4 | 10.4 | 6.0 | 6.2 | 25.6 | 26.2 |

**Table II.** Type I errors (500 samples) for the simulation with the distinct missing data pattern
(20% missing data)

| | REML $\chi^2$ | REML F | Imputation |
|---|---|---|---|
| Treatment | 13.8 | 7.0 | 18.6 |
| Time | 11.4 | 6.6 | 38.6 |
| Treatment X Time | 11.2 | 7.0 | 35.6 |

The Expected Mean Squares (calculated using the method described in the Appendix) under the null hypotheses after imputation are then:

$E(MS\alpha) = 1.80\,\sigma^2_\varepsilon + 9.24\,\sigma^2_{\alpha\pi} + 0.48\,\sigma^2_{\beta\pi}$

$E(MS\beta) = 1.60\,\sigma^2_\varepsilon + 0.28\,\sigma^2_{\alpha\pi} + 2.56\,\sigma^2_{\beta\pi}$

$E(MS\alpha\beta) = 1.60\,\sigma^2_\varepsilon + 0.28\,\sigma^2_{\alpha\pi} + 0.56\,\sigma^2_{\beta\pi}$

$E(MS\alpha\pi) = 0.68\,\sigma^2_\varepsilon + 3.42\,\sigma^2_{\alpha\pi} + 0.19\,\sigma^2_{\beta\pi}$

$E(MS\beta\pi) = 0.76\,\sigma^2_\varepsilon + 0.11\,\sigma^2_{\alpha\pi} + 1.26\,\sigma^2_{\beta\pi}$

$E(MS\varepsilon) = 0.76\,\sigma^2_\varepsilon + 0.11\,\sigma^2_{\alpha\pi} + 0.22\,\sigma^2_{\beta\pi}$

These expectations may be compared to the complete data Expected Mean Squares given previously with u=7 and t=2. This data pattern was subjected to a simulation as well and the resulting type I errors are presented in Table II. While the REML approach is unaffected, Imputation leads to clearly unacceptable results. In a situation with a missing data pattern like this, one would obviously be extremely careful in drawing conclusions. Nevertheless, the example demonstrates the inadequacy of the imputation method in general.

**Example**

In a study performed at our Centre [27] the effects of different infusion rates of Felodipine (a drug used in the treatment of high blood pressure) on the circulatory system were investigated. Previous publications suggested that the speed at which a drug like this enters the body may influence its effects. Slowly increasing concentrations would give regulatory mechanisms time to adjust, while an abrupt change might cause an exaggerated response.

Four different treatments were investigated: 1. a fast infusion of Felodipine reaching a plateau in 20 min and remaining there for 8 hours, 2. a slow infusion of Felodipine taking 8 hours to reach the same plateau, 3. an infusion with only the vehicle used for dissolving the Felodipine which was suspected of having haemodynamic effects, and 4. an infusion with saline acting as Placebo. These four treatments were assigned to 8 healthy male volunteers according to two different 4X4 Latin squares balanced for first order carry-over effects. A wash-out period between treatments of one week was incorporated.

Regulation of blood pressure is in part performed by the baroreceptor-reflex which induces changes in heart rate to counteract changes in blood pressure. Changes in baroreceptor sensitivity that could account for a change in response, were assessed using the Valsalva manoeuvre. During this manoeuvre subjects are required to forcefully expire into a tube connected to a manometer during 20 seconds, which will induce a change in blood pressure. Heart rate and blood pressure are measured continuously.

**Table III.** Results for the analysis of the example (Valsalva data). For REML results ÷5 values are divided by their Df for comparative purposes.

| | REML | | | Imputation | | Df |
|---|---|---|---|---|---|---|
| | 'F' | p-val $\chi^2$ | p-val (F) | F | p-val | |
| Treatment (Overall) | 1.20 | 0.307 | 0.333 | 1.25 | 0.315 | 3,21 |
| slow vs fast | 0.64 | 0.425 | 0.450 | 0.75 | 0.415 | 1,7 |
| vehicle vs saline | 0.14 | 0.706 | 0.719 | 0.15 | 0.706 | 1,7 |
| *felo* vs *no_drug* | 2.84 | 0.092 | 0.136 | 3.22 | 0.116 | 1,7 |
| Time (Overall) | 0.40 | 0.880 | 0.875 | 0.31 | 0.929 | 6,42 |
| Treatment X Time (Overall) | 1.67 | 0.037 | 0.053 | 2.01 | 0.014 | 18,126 |
| slow vs fast X Time | 0.39 | 0.884 | 0.879 | 0.57 | 0.755 | 6,42 |
| vehicle vs saline X Time | 1.79 | 0.097 | 0.124 | 2.17 | 0.065 | 6,42 |
| *felo* vs *no_drug* X Time | 2.72 | 0.012 | 0.026 | 3.03 | 0.015 | 6,42 |

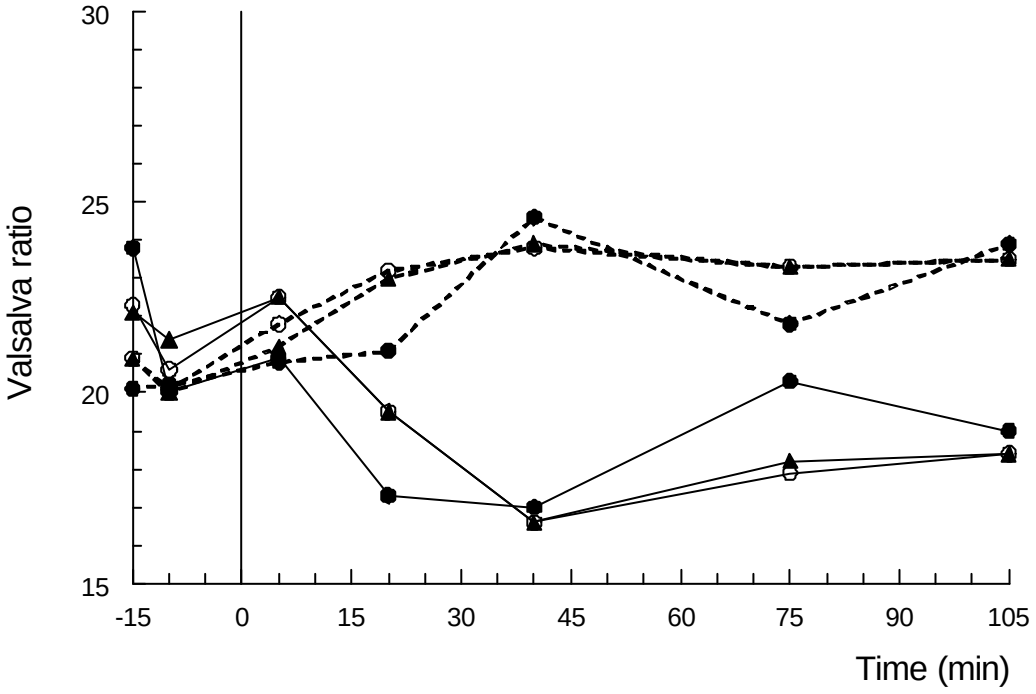


**Figure 1.** Time course of Valsalva ratio for the *felo* (solid line) and *no_drug* (dashed line) contrasts; All infusions start at 0 min. open dot=REML estimates, triangle=estimates using Imputed values, closed dot=available case averages

The 'Valsalva ratio' resulting from this procedure is defined as the maximum increase in Heart period divided by the maximum increase in Mean Arterial Pressure. However, this procedure does not always produce interpretable measurements and therefore a relatively large number of data (7.1% in the first two hours) were missing. At the time no method was available for the handling of missing data, so we decided to analyse the average of available cases in the first two hours after start of the infusion (5 time points) thereby losing information on the time-profile, which was regrettable since change in baroreceptor sensitivity over time was a main subject of study. The averages were analysed using orthogonal contrasts; fast versus slow, vehicle versus saline, and *felo* (average of fast and slow) versus *no_drug* (average of vehicle and saline). No significant differences in sensitivity were seen between the slow and fast treatments or between saline and vehicle; only the *felo* versus *no_drug* contrast was significant (p=0.047).

The data, this time including the two pre-treatment values, are re-examined using the techniques described in this paper and results are presented in Table III. REML estimation demonstrates a marginally significant overall treatment by time interaction attributable to the *felo* versus *no_drug* contrast (p=0.026 compared to $F_{[6,42]}$). None of the other contrasts are significant. Imputation leads to comparable results, only p-values are smaller (since F-values are larger). Subsequent examination of comparisons per time-point can be performed in at least 3 different ways; using paired t-tests on available cases, using REML estimation per time-point or using paired t-tests with the missing values imputed. The estimates for the *felo* versus *no_drug* contrasts using these 3 methods are given in Figure 1 and seem to indicate that REML and Imputation estimates are rather similar while available case t-tests (having at least 5 complete cases per time-point) produce different estimates. The contrast at 40 min. post infusion was significant for all 3 methods while the 20 min. contrast was significant for the Imputation t-test only; in view of the described liberal nature of Imputation methods this 'significance' should be regarded with caution.

## Discussion and concluding remarks

Imputation in the form presented in this paper is unacceptable as a general method. Its only use is perhaps for very small fractions of missing data (less than 5%) in which case any ad-hoc method will probably perform equally well. Imputation might be improved by calculating the Expected Mean Squares and using these to compute synthetic F-values with more acceptable properties [9]. However, without special software this may be a cumbersome procedure and acceptability would still be an issue. Imputation may also be improved by adding random noise to the values to be filled in. This may alleviate some of the problems with the inflated F-values. Further improvement could be obtained by repeating this stochastic imputation process several times and combining the results into a single probability statement [28]. However, all of these enhancements will take Imputation further away from its original appeal of simplicity. Its use should therefore be restricted to those fields where

likelihood based analyses have not yet been implemented or where its implementation is unfeasible.

On the basis of both theory and simulation results, REML methodology is the choice of preference. Data are only required to be 'missing at random' while employment of F-distributions for assessing significance of the Wald statistics in small sample situations leads to adequate results. The methodology can even be seriously considered for routine analysis of complete data, since it provides a powerful tool for the handling of situations in which the standard assumptions about the covariance matrix are either unrealistic or unwanted.

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## Appendix

The variance-covariance matrix for the univariate Repeated Measures design in BMDP5V has to be specified by the user as the linear combination of the four matrices defining the four variance parameters $\sigma^2_{\pi}$, $\sigma^2_{\beta\pi}$, $\sigma^2_{\alpha\pi}$, $\sigma^2_{\varepsilon}$. A specific example for 2 treatment and 3 time levels is:

```
/input var = 6.
file = 'test_rm.asc'.
format = free.
/var names = A1B1 A1B2 A1B3 A2B1 A2B2 A2B3. missing = 6 * -99.
/design dpname = Y.
dpvar = A1B1 A1B2 A1B3 A2B1 A2B2 A2B3.
repeated = A, B.
levels = 2, 3.
/model Y = 'A*B'.
/structure type = linear.
 nparm = 4.
 matrix(1)=
1,
1,1,
1,1,1,
1,1,1,1,
1,1,1,1,1,
1,1,1,1,1,1.
 matrix(2) =
1,
1,1,
1,1,1,
0,0,0,1,
0,0,0,1,1,
0,0,0,1,1,1.
 matrix(3) =
1,
0,1,
0,0,1,
1,0,0,1,
0,1,0,0,1,
0,0,1,0,0,1.
 matrix(4) =
1,
0,1,
0,0,1,
0,0,0,1,
0,0,0,0,1,
0,0,0,0,0,1.
 initial = 25, 5, 1, 10.
/compute algor = reml.
/end
```

Initial estimates for the variance parameters have to be given but do not seem very critical.

*Calculation of Expected Mean Squares for RM-ANOVA after Regression Imputation*

The first part of this derivation can also be found in Kshirsagar and Deo [20]. Consider the situation where $Y$ is the data vector and $X$ the design matrix. $Y$ and $X$ are partitioned into a part with the data present ($Y_p$ and $X_p$) and a part with the data missing ($Y_m$ and $X_m$). The values for imputation minimising the Residual Sum of Squares ($Y^*$) are found by:

$$Y^* = (I - H_{mm})^{-1} H_{mp} Y_p$$

where

$$H_{ij} = X_i (X^T X)^{-1} X_j^T$$

Next we construct the matrix $B$ which calculates the values used for the RM-ANOVA ( $\hat{\imath}$ ) from the data present ($Y_p$) :

$$\hat{Y} = \begin{pmatrix} Y_p \\ Y^* \end{pmatrix} = \begin{pmatrix} I \\ (I - H_{mm})^{-1} H_{mp} \end{pmatrix} Y_p = B Y_p$$

The Sums of Squares are calculated by $\hat{\imath} A_i \hat{\imath} = Y_p^T B^T A_i B Y_p$ where $A_i$ is the quadratic form defining the contrast (e.g. Rawlings [26]). The expectation of the quadratic form $YAY$ where $Y \cong N(0,\Sigma)$ is given by $tr(A\Sigma)$. The Expected Mean Squares after imputation are therefore given by :

$$EMS_i = tr(B^T A_i B \Sigma)$$

Chapter 3

# Estimating impossible curves using NONMEM

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**Summary**

On fitting model equations to experimental data, the situation may arise that individual subjects provide insufficient information to obtain adequate parameter estimates due to the fact that not all aspects are exhibited by all subjects or that the models are simply too complex. This may be solved by applying nonlinear mixed effect modelling to the data, which integrates the information provided by different subjects.

We aim to provide insight into the methodology and its use in these situations, illustrated by three examples: determination of pharmacokinetics in a rising dose design, where the lower doses provide insufficient information (due to assay limitations) to estimate terminal half-life; determination of the kinetics of the low molecular weight heparin enoxaparine (Clexane®) using anti-Xa activity, effectively dealing with lingering low/basal activity; simultaneous estimation of the pharmacokinetics and pharmacodynamics of the low molecular weight heparin dalteparine (Fragmin®) after subcutaneous and intravenous administration.

## Introduction

Models describing the time-course of a treatment or drug are an integral part of the clinical pharmacologist's toolbox; compartmental models are well established in pharmacokinetics, and pharmacodynamic models find increasing use [1]. In the course of new drug development, increasing emphasis is placed on using and integrating pharmacokinetic and pharmacodynamic information resulting in a more rational drug development strategy [2, 3]. Intensive sampling in a controlled research setting will often enable accurate estimation of the relevant model parameters. However, the information needed for adequate parameter determination may be present in only some of the subjects, or models may have too many parameters and become too complex to allow accurate parameter estimation in individual subjects. This paper presents a solution to this problem by using nonlinear mixed effect modelling as a means for combining information from different individuals while preserving individuality. After a gradual development of the theoretical background, three examples will be used to illustrate uses and usefulness of the methodology.

## Nonlinear mixed effect modelling

### *Theory*

At the basis of nonlinear mixed effect modelling lies a nonlinear mathematical model describing the response of an individual. Take as an example the one-compartment kinetic model describing the concentration-time profile of a drug after an intravenous injection:

$$C_j = \frac{D}{V} e^{-kt_j} \qquad (1)$$

The equation predicts concentrations ($C_j$) as a function of time ($t_j$), dose ($D$) and the two parameters $V$ (Volume of distribution) and $k$ (elimination rate constant). It can be written in a general form using the following expression:

$$\hat{Y}_j = f(x_j, \boldsymbol{\psi}) \qquad (2)$$

where the response $Y$ is predicted by $\hat{\imath}$ using the unspecified function $f$. $f$ is a function of the known quantities $x$ (dose $D$ and time $t_j$ in (1)), and the unknown parameters $\boldsymbol{q}$ ($k$ and $V$ in (1)) which are to be estimated. The predicted response $\hat{\imath}$ will usually deviate from the actual observations. The link between the structural model (2) and the observations is provided by the intra-individual error model which quantifies the deviations of model predictions from actual observations. The two most common intra-individual error models are the additive normal error model and the multiplicative log-normal error model. For the additive normal error model, the observed response $Y$ is given by:

$$Y_j = f(x_j, \boldsymbol{q}) + \boldsymbol{e}_j \qquad (3)$$

where the individual deviations from the predicted response $\varepsilon_j$ are assumed to follow a normal distribution with mean 0 and variance $\sigma^2$. This model is a natural choice if each measurement is assumed to be equally precise for all values of $Y_j$. This is usually the case in concentration-effect modelling.

For the multiplicative log-normal error model the observed response $Y$ is given by:

$$Y_j = f(x_j, \boldsymbol{q}) \bullet e^{e_j} \qquad (4)$$

where $e^{e_j}$ is assumed to follow a log-normal distribution with median 1 and constant coefficient of variation CV. The log-normal error model is often appropriate if measurements can only be positive and if they become less precise when the measured value increases; the variance of the deviations is proportional to the square of the predicted response. This is often the case with pharmacokinetic models. Other error models may be used in practice such as a combination of the additive and log-normal error model to improve prediction at the lower limit of assay precision where variance may be assumed constant, and the power function model where the variance of the deviations is assumed proportional to some power (which is to be estimated) of the predicted response.

Models (3) and (4) which describe the response of a single subject, can be estimated using ordinary nonlinear regression, resulting in intra-individual variability estimates ($\sigma_\varepsilon$ or $CV_\varepsilon$), estimates for the parameters $\boldsymbol{q}$ (such as terminal half-life or $EC_{50}$) and approximate standard errors (SEMs) indicating the precision of the estimated $\boldsymbol{q}$s.

Nonlinear mixed effects modelling expands the single-subject model by simultaneously estimating the curves for all subjects. The multiple subjects extension of (4) becomes:

$$Y_{ij} = f(x_{ij}, \boldsymbol{q}_i) \bullet e^{e_{ij}} \qquad (5)$$

where $Y_{ij}$ indicates the response from subject i at time j, and the $\phi_i$ are the individual parameters belonging to subject i. However, it is not the individual parameters that are estimated but rather their mean and variance. These correspond to the population average parameters and the inter-individual variability in these parameters. The individual parameters $\phi_i$ can be modelled as:

$$\phi_i = \phi \bullet e^{\eta_i} \qquad (6)$$

where $\phi$ represents the population average parameters, and the $\eta_i$s quantify individual deviations from these population averages. Linearisation of (6) (a computational necessity) results in a statistical model for which the square root of the estimated variances of the $\eta_i$s provides coefficients of variation (CVs) for inter-individual variability in the $\phi$s.

The term 'mixed-effect modelling' is the statistical term used in the situation where a combination of fixed and random effects is studied. The random effects (the $\eta_i$s from equation (6)) quantify the inter-individual variabilities in the kinetic/dynamic parameters. The fixed effects (the $x_{ij}$s from equation (5)) are part of the experimental design and under control of the investigator such as the time of measurement or the treatment applied. Nonlinear mixed effect modelling allows the simultaneous estimation of the same nonlinear structural model in all subjects. This does not mean that all subjects are forced to be the same, because their parameter values are allowed to vary. The power of the technique lies in the ability to combine the information from all subjects while preserving the individuality. If part of the curve is missing for one subject, then the information that is available for that subject may be combined with information obtained from the other subjects to provide a reasonable estimate for the missing part of the curve.

*Estimation*

Because of the nonlinear nature of nonlinear mixed effect modelling, exact and analytic results are unavailable, and the problem must be approached through approximations and by the use of iterative techniques. The most extensively developed and widely used is a first-order Taylor approximation which results in a linearisation of equation (5) while successive iterations are evaluated at the mean value of the $\varsigma_i$s. This means that during a step in the search for the best parameter combination, all subjects have the same parameter values. This approximation works well if subjects provide only little information. This so called 'first-order method', was first implemented in the NONMEM software program [4]. A more accurate approximation to equation (5) is obtained if parameter values are calculated for each individual during each step of the parameter search. This method was investigated by Lindstrom and Bates [5] and also implemented in NONMEM as 'conditional' estimation. Other extensions and alternatives have been proposed, investigated and implemented. Sheiner and Ludden [6] provide a good

introduction to population methods. Applications are numerous, some of which can be found in the *British Journal of Clinical Pharmacology* [7, 8]. An exhaustive bibliography with methodological references and references to applications is provided by Yuh *et al* [9].

The core of the NONMEM program is a set of subroutines written in the FORTRAN programming language, which are linked with the model-defining subroutine to produce the NONMEM executable file. Because of this architecture, NONMEM can run on any platform supporting a FORTRAN compiler. It is equipped with a module for model and data definition called NMTRAN and comes with an extensive library of model-defining subroutines for pharmacokinetic applications called PREDPP. Models may be modified, and models not included in the library can be defined by the user.

*From population to individual; empirical Bayes estimates*

In order to assess adequacy of parameter estimates resulting from a NONMEM analysis, it is essential to be able to go back from the population estimates to the individual. Although nonlinear mixed effect modelling itself does not provide individual $\eta_i$ s, the NONMEM program has the capability for calculating empirical Bayes estimates. Bayes estimates are calculated conditional on prior specified information e.g. individual clearance is estimated in accordance with the mean and variability of population clearance. The estimates are empirical in this case because the prior information is not obtained from some external source but is derived from the data to be analysed. In this context, empirical Bayes estimates are parameter estimates for the individual subject that take the previously obtained population information into account. The result is a weighted combination of individual and population information where the weighting depends on how much information the individual itself supplies and how large the inter-individual variability was estimated to be. If a subject in a group has a large number of accurate data points the empirical Bayes estimates for that subject will largely be determined by that subject alone. Conversely, empirical Bayes estimates for a subject with only a few data points will largely be determined by the information obtained from the other subjects (the population estimates). Therefore, empirical Bayes estimates allow information to be borrowed from other subjects if necessary. NONMEM can calculate individual predicted concentration or effect time-profiles based on these estimates.

The empirical Bayes estimates are extremely valuable in assessing model-fit and adequacy of convergence, and greatly increase the confidence in the final NONMEM results. Nonlinear mixed effect modelling is a numerically complex technique which is highly dependent on good initial estimates and may converge to unsatisfactory parameter estimates. The calculation of the empirical Bayes estimates is far less fraught with numerical difficulties. Therefore, if average empirical Bayes estimates are very different from the NONMEM population estimates, this is an indication that something is wrong: NONMEM may have inadequately converged or the structural or error model may have been misspecified.

Alternatively, the mathematical complexity of the model may require a more accurate approximation, e.g. by undertaking 'conditional' estimation instead of the 'first order method'. Conversely, if empirical Bayes estimates correspond well with the NONMEM results, and the individual time profiles look adequate, NONMEM estimates may be presented with confidence.

The empirical Bayes estimates should not be used for subsequent statistical analyses; they are no longer independent estimates because they borrow information from each other. Their variability is generally somewhat lower than the NONMEM CVs indicate because they are too much alike, and if NONMEM cannot estimate inter-individual variability the calculated empirical Bayes estimates are identical for all subjects.

A global measure for the goodness of fit is provided by the minimum value for the objective function. Competing models may be compared on the basis of these values; the model with the lowest minimum value provides the best fit to the data.

## Methods

All calculations for this paper were performed on a 486DXII 66 MHZ IBM compatible personal computer running under MS-DOS using the Microsoft FORTRAN PowerStation 1.0 compiler with NONMEM Version IV, NMTRAN Version II and PREDPP Version III. NONMEM control files used to analyse the examples can be found in the appendix. All examples were initially analysed using the 'first order method', with a final run using the 'conditional method'. Inter-individual variability in parameters was always modelled using the constant coefficient of variation model (6).

**Example 1; A rising dose design**

*Problem*

In a study to determine kinetics and dynamics of a novel anti-hypertensive drug, 18 patients were investigated in a rising dose design (unpublished data). The patients were taken off their own medication until diastolic blood pressure was between 100-115 mmHg. Each patient was seen on three occasions in which active drug (twice) or matching placebo (once) was given as an IV bolus. The three panels of subjects contained doses of 1 and 3mg, 3 and 10mg, and 10 and 30mg of active drug. The higher dose was always given after the lower dose (if well tolerated), with the placebo on a random occasion.

Serious problems were encountered when individual parameters at lower doses were estimated with conventional nonlinear regression because the terminal part of these curves is missing due to assay limitations. These lower-dose curves already have a tendency to level off without providing sufficient information to adequately estimate the terminal part of the curve.

*Solution*

NONMEM enables fitting a two compartment open model to all data simultaneously. By assuming constant pharmacokinetic parameters in a subject for the two different doses, the amount of information per subject effectively doubles resulting in more stable parameter estimates. The two-compartment open model after an IV bolus is usually represented using the equation:

$$C_j = A e^{-\alpha t_j} + B e^{-\beta t_j} \qquad (7)$$

Parameters $A$ and $B$ themselves are essentially meaningless but can be re-expressed using administered dose ($D$), clearance ($Cl$) and volume of the central compartment ($V_c$) resulting in the following equation:

$$C_j = \frac{D}{V_c(\beta - \alpha)Cl}\left[ (\alpha\beta V_c - \alpha Cl) e^{-\alpha t_j} - (\alpha\beta V_c - \beta Cl) e^{-\beta t_j} \right] \qquad (8)$$

Initial and terminal half-life are calculated as $t_{1/2\alpha} = \ln 2/\alpha$ and $t_{1/2\beta} = \ln 2/\beta$.
A log-normal intra-individual error distribution was assumed as is usual for pharmacokinetic data, which due to NONMEM's linearisation is approximated with a constant coefficient of variation model. NONMEM results are presented in table I and concentration-time profiles for

subjects 1 (1 and 3mg) and 13 (10 and 30mg) using their empirical Bayes estimates are presented in figure 1.

**Table I.** NONMEM estimates for the anti-hypertensive drug

| | Mean | SEM | CV |
|---|---|---|---|
| $t_{1/2\alpha}$ (min) | 90.7 | 8.37 | 13.4% |
| $t_{1/2\beta}$ (min) | 362 | 155 | 52.6% |
| $V_c$ (l) | 4.80 | 0.232 | 13.9% |
| Cl (ml•min$^{-1}$) | 32.7 | 1.47 | 16.8% |
| CV residual error | 23.1% | | |

Mean=population average, SEM=approximate standard error of the population average, CV=inter-individual coefficient of variation, $t_{1/2\alpha}$=initial half-life, $t_{1/2\beta}$ =terminal half-life, $V_c$=volume of distribution of the central compartment, Cl = clearance

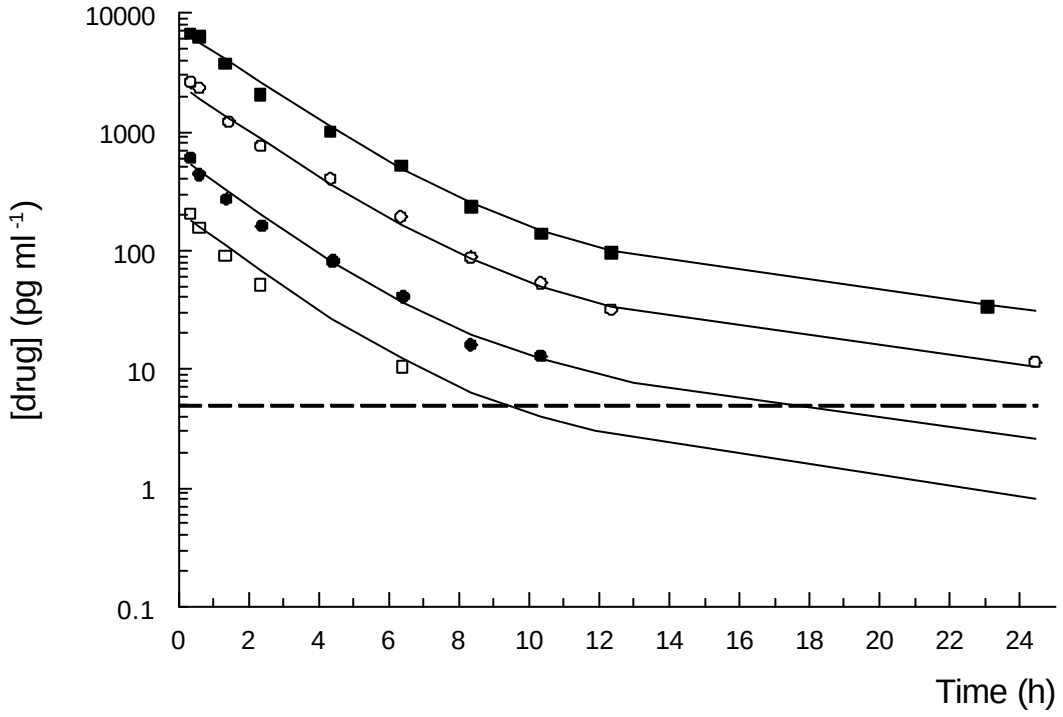


**Figure 1.** Predicted and observed individual concentration profiles of the anti-hypertensive drug using NONMEM estimation and empirical Bayes estimates. The horizontal line (----) indicates the assay detection limit. **G** 1mg, subject 1; **M** 3mg, subject 1; **F** 10mg, subject 13; **O** 30mg, subject 13.

The graph illustrates that parameter estimates can be obtained that provide an acceptable description of the time-profile with extrapolation to beyond the point were measurements could be obtained. All data can be described using the same model and, by borrowing elimination half-life information from higher doses, sensible parameter estimates can be obtained for the lower doses. The low doses may not provide all the information but may nevertheless serve to examine the presence of dose-dependent kinetics. The estimates and individual graphs provide no indication of dose-dependency. By introducing an extra slope parameter, clearance can be written as a linear function of administered dose. This slope was estimated at 0.011 with an approximate 95% confidence interval of (-0.024 / 0.046) which translates into a non-significant 10% increase in clearance from the 1mg to the 30mg dose.

Although no indications can be found for dose-dependency, these results should be viewed with caution. The large SEM for the terminal half-life estimate indicates that there is very little information to accurately determine this parameter, which is of course essential if a definitive statement about dose-dependency is to be made. Nonlinear mixed effect modelling is not a panacea for generating information that is simply not present.

**Example 2; Low molecular weight heparin kinetics**

*Problem*

In a study comparing the anti-clotting effects of four heparin-like substances [10] (dalteparine (Fragmin®), nadroparine (Fraxiparine®), enoxaparine (Clexane®) and danaparoï d (Orgaran®)), 12 subjects received IV administrations of all four drugs on separate occasions in an open randomised cross-over design. These drugs are actually a mixture of substances and this complicates kinetic assessment because a simple concentration cannot be obtained. The effect these drugs have on the coagulatory system, measured for instance by the activity of clotting factor Xa, is generally used as a surrogate concentration measure.

With standard nonlinear regression of the Clexane® results, five subjects could be adequately described by a mono-exponential function, four required a bi-exponential function and three provided no adequate fits at all. The data points and dotted lines in figure 2 illustrate a 'one-compartment subject' and a 'two-compartment subject' with their ordinary nonlinear regression fits.

*Solution 1*

This situation could be viewed as analogous to example 1, in which information on the second compartment is missing for some of the subjects. The two-compartment open model as defined by equation (8) was applied to these data. Table II provides the results which are illustrated in figure 2 by the continuous lines. These indicate that subjects which were formerly described by a one-compartment model can be described by a two-compartment model if information on the second compartment is borrowed from the other subjects. This enables a more cohesive presentation and interpretation of results. The coefficient of variation of zero for the clearance estimate indicates that insufficient information is present to correctly estimate the inter-individual variability in all the parameters. Clearance and terminal half-life are tightly linked; the constant clearance is compensated in individual subjects by a large variability in terminal half-life. Forcing a coefficient of variation of zero on terminal half-lives resulted in an equally good fit (as indicated by similar minimum values for the objective functions) with a variability estimate for clearance of 34%.

*Objections*

The original data had to be modified to allow analysis. Activities of clotting factors are used as a concentration surrogate, but low activity may also be endogenous. Non-zero pre-values were therefore subtracted from the activity measurements and a limit of quantitation was imposed below which measurements were considered absent. It could be argued that the lingering low activity necessitating a second compartment was actually the prevailing basal activity of the system.

*Solution 2*

Another approach to these data is to implement the constant presence of basal activity by adding a constant (to be estimated) to the original kinetic model:

$$C_j = base + \frac{D}{V} e^{-kt_j} \qquad (9)$$

This way, the data may be analysed without pre-value subtraction or limits of quantitation, and a one-compartment model might suffice.

**Table II.** NONMEM estimates for Clexane[7] anti-Xa activity using a two-compartment model

| | Mean | SEM | CV |
|---|---|---|---|
| $t_{2\alpha}$ (min) | 120 | 5.43 | 11.7% |
| $t_{2\beta}$ (min) | 4780 | 722 | 63.5% |
| $V_c$ (l) | 6.01 | 0.254 | 11.3% |
| Cl (ml/min) | 9.44 | 0.418 | 0.0% |
| | | | |
| CV residual error | 11.8% | | |

Mean=population average, SEM=approximate standard error of the population average, CV=inter-individual coefficient of variation, $t_{2\alpha}$=initial half-life, $t_{2\beta}$=terminal half-life, $V_c$=volume of distribution of the central compartment, Cl=clearance

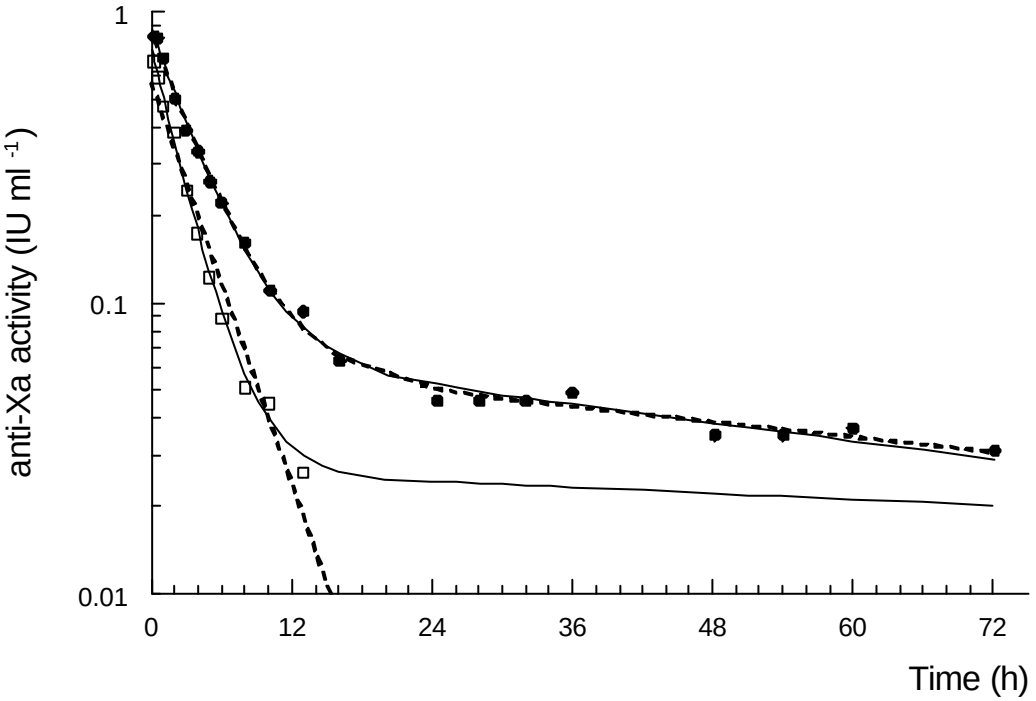


**Figure 2.** Predicted and observed anti-Xa activities after Clexane[7] IV using ordinary nonlinear regression (dashed line) with a one-compartment (subject 4) or two-compartment (subject 5) model and using NONMEM empirical Bayes estimates (solid line) with a two-compartment model. **G** subject 4; **M** subject 5.

**Table III.** NONMEM estimates for Clexane[7] anti-Xa activity using a one-compartment model with basal activity

| | Mean | SEM | CV |
|---|---|---|---|
| $t_2$ (min) | 130 | 4.49 | 8.6% |
| Cl (ml/min) | 32.9 | 1.69 | 15.1% |
| base (IU/ml) | 0.0254 | 0.00333 | 37.7% |
| | | | |
| SD residual error | .0274 | | |

Mean=population average, SEM=approximate standard error of the population average,
CV=inter-individual coefficient of variation, $t_2$=half-life, Cl=clearance, base=basal activity

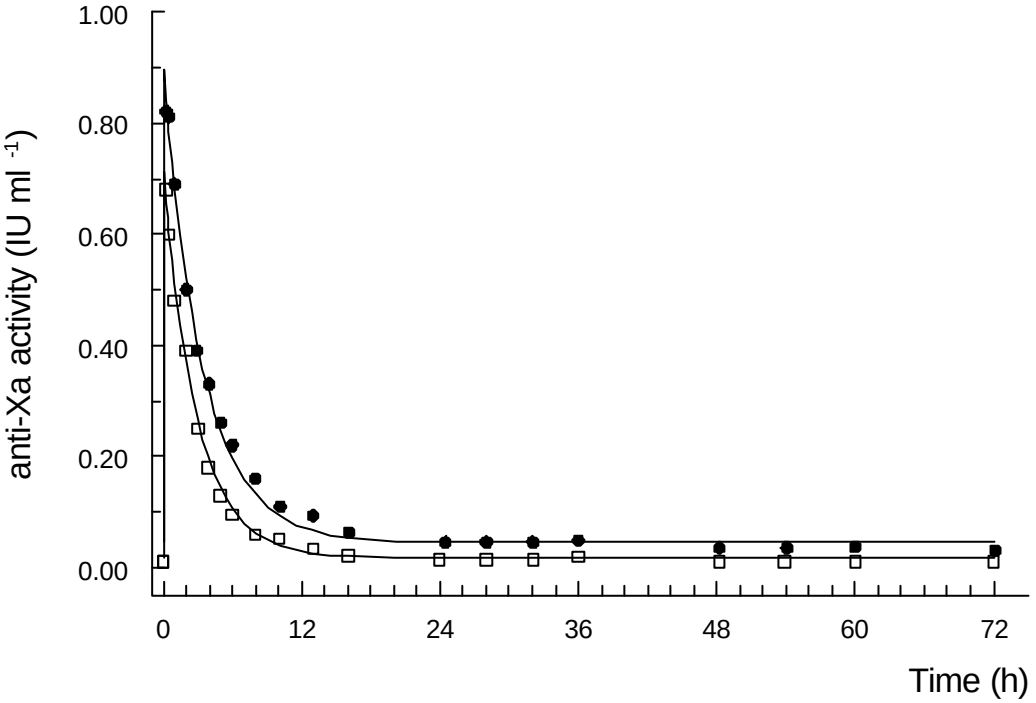


**Figure 3.** Predicted and observed anti-Xa activities after Clexane[7] IV using empirical Bayes estimates and a one-compartment model with basal activity. **G** subject 4; **M** subject 5.
The anti-Xa axis is not on a log-scale because an additive error model was assumed (see text).

Examination of residual plots (deviations of actual data from model predictions using empirical Bayes estimates, plotted against actual observations) indicate that with these data the additive normal error model (equation (3)) is more appropriate than the multiplicative log-normal error model (equation (4)). This is caused by the fact that the anti-Xa assay is not very accurate in the lower concentration range and therefore model deviations do not decrease at lower concentrations as is usually the case with kinetic data.

The NONMEM results are presented in table III and are illustrated by figure 3 with graphs of subjects 4 and 5. These indicate that a one-compartment model with the basal activity parameter may provide an adequate description of the anti-Xa activity time course. The estimated half-life agrees with the initial half-life estimate obtained with the two-compartment model. The clearance however is about three times as high (two-compartments: 9.44 ml/min *vs* one-compartment: 32.7 ml/min). This is caused by the fact that with the two-compartment model, a substantial part of the area under the curve is attributed to the second compartment which is assumed absent in the one-compartment case. In the original article [10], the authors present a clearance value of 26.7 ml/min obtained by dividing the dose by the calculated AUC up till the last available measurement (at 24 hours). The fact that this intuitively plausible clearance estimate matches the one-compartment result, combined with the accepted practice of subtracting non-zero pre-values which is largely analogous to estimating a basal activity, favours the one-compartment basal activity model. The two different approaches cannot be compared using objective function values (as one would usually do with competing models) because they are not applied to the same data set; the pre-value subtraction needed for solution 1 generates an essentially different set of data. It is not possible on the basis of the data alone to come to an objective conclusion about which model and which clearance estimate is the 'right' one. The true test as to which clearance estimate is most appropriate lies in multiple dosing/continuous infusion pharmacokinetics experiments; the predicted average steady state concentration varies three-fold between the two competing models.

## Example 3; Intravenous and subcutaneous pharmacokinetics and pharmacodynamics

*Problem*

In a study performed to compare the kinetics and dynamics of four different drugs with anti-coagulatory effects [11] (dalteparine (Fragmin®), nadroparine (Fraxiparine®), danaparoï d (Orgaran®) and heparin) 12 subjects were randomised to eight different occasions each, on which one of the drugs was administered either by subcutaneous (SC) or by intravenous (IV) route. Anti-Xa activity was measured as was the Activated Partial Thromboplastin Time (APTT), a more global measure of anti-coagulatory effect.

One of the challenges in these data lies in combining information from intravenous and subcutaneous administration to obtain estimates of bio-availability and absorption. If only

subcutaneous data were available no estimate could be obtained for bio-availability, clearance or volume of distribution. Absorption and elimination half-lives could not be estimated accurately unless they were very different because the processes of absorption and elimination cannot be separated very well without independent information. The same curves can be produced with a wide range of elimination and absorption values because an increase in absorption can be compensated by an increase in elimination resulting in the same curve.

The APTT data could be analysed using the one-compartment model with basal activity, in analogy with the anti-Xa data from example 2. This would provide an effect profile description using the kinetic idiom. On the other hand, if anti-Xa activity is viewed as a concentration measure and APTT as a dynamic response, then these data lend themselves to a simultaneous pharmacokinetic/pharmacodynamic (PK/PD) analysis providing additional information on the concentration-effect relationship between anti-Xa activity and APTT response.

*Solution-kinetics*

The anti-Xa data were analysed using the basal activity model with one-compartment and (for Orgaran[7]) two-compartment kinetic models. The kinetics/dynamics of one of the low molecular weight heparins (Fragmin[7]) is presented as an illustration.

Far more accurate rate of absorption estimates can be obtained by pinning down elimination half-life using IV information. Because the same subject received both IV and SC treatments, the two routes could be simultaneously estimated. Common parameters (elimination half-life, clearance, basal activity) were assumed the same for both routes in individual subjects.

This complex situation was advanced in steps to ensure successful convergence. First the IV data alone were analysed. Then all data were analysed together but the parameter estimates obtained from the IV fits were fixed to obtain good estimates of bio-availability and absorption. A final analysis was run in which no parameters were restricted.

*Solution-dynamics*

A first attempt at investigating the concentration-effect relationship is made using the previously obtained empirical Bayes estimates to describe the anti-Xa profile. By plotting predicted anti-Xa measurements against APTT data for each individual, an appropriate concentration-effect model may be selected. Initially, a linear model seemed appropriate for these data.

The empirical Bayes estimates can be presented to the NONMEM program as data, along with the dynamic measurements (see NONMEM Manual V p 129-130). This keeps the kinetic part of the analysis fixed and allows a first estimation of the concentration-effect relationship parameters. The obtained precision estimates for these parameters (the SEMs) are too optimistic however because variability in the kinetic profile is not accounted for. A final analysis is therefore performed in which the kinetic and the dynamic part are no longer restricted.

Close examination of the estimated individual concentration-effect relationships revealed that a slightly curvilinear relationship would be more appropriate. Because there was no indication of a maximum effect, a somewhat unconventional exponential model was implemented which estimates the parameter $I_{10}$. This is the anti-Xa activity increase needed to obtain a 10% increase in APTT:

$$APTT_j = APTT_0 \, e^{\frac{\ln(1.1)}{I_{10}} Xa_j} + \varepsilon_{2j} \qquad (10)$$

The measured APTT at time j ($APTT_j$) is a function of the anti-Xa activity at time j ($Xa_j$), the basal APTT level ($APTT_0$), the $I_{10}$ parameter, and the additive residual $\varepsilon_{2j}$. The "2" indicates that this residual error is different from the intra-individual anti-Xa kinetics residual, requiring a separate variance parameter. Table IV provides the obtained parameter estimates and figure 4 illustrates the result for subject 3. In a situation as complex as this you need all the information you can get. The use of nonlinear mixed effect modelling allows you to obtain the best possible information on both kinetics and dynamics of the drug under the two modes of administration.

**Table IV.** NONMEM estimates for Fragmin[7] anti-Xa activity after intravenous and subcutaneous administration using a one-compartment model with basal activity, and the anti-Xa - APTT relationship estimates using an exponential concentration-effect model.

| | Mean | SEM | CV |
|---|---|---|---|
| $t_{2}$ E (min) | 92.7 | 6.07 | 8.3% |
| $t_{2}$ A (min) | 76.6 | 5.19 | 0.0% |
| Cl (ml/min) | 46.1 | 3.37 | 14.1% |
| F (%) | 70.5 | 7.81 | 34.8% |
| base (IU/ml) | 0.0192 | 0.00271 | 16.6% |
| $APTT_0$ (sec) | 30.2 | 0.910 | 10.6% |
| $I_{10}$ (IU/ml) | 0.0665 | 0.00439 | 19.6% |
| | | | |
| SD residual error anti-Xa vs time | | 0.0207 | |
| SD residual error APTT vs anti-XA | | 1.70 | |

Mean=population average, SEM=approximate standard error of the population average,
CV=inter-individual coefficient of variation, $t_{2}$ E=elimination half-life, $t_{2}$ A=absorption half-life, Cl=clearance, F=bioavailability, base=basal activity, $APTT_0$ = basal APTT level,
$I_{10}$ = anti-Xa increase required to induce a 10% increase in APTT

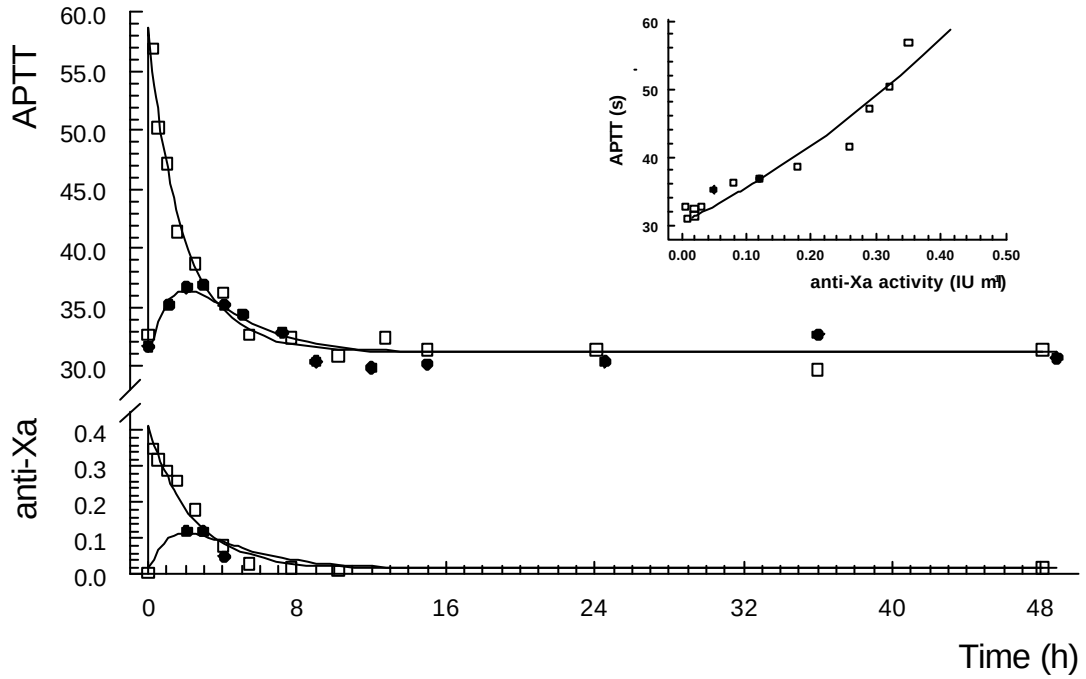


**Figure 4.** Predicted and observed anti-Xa and APTT values and the concentration-effect relationship for subject 3 after Fragmin[7] administration using empirical Bayes estimates, a one-compartment model with basal activity, and an exponential concentration-effect relationship.
**G** intravenous administration; **M** subcutaneous administration.

## Discussion and conclusions

The original field of application of the NONMEM program is in determining population pharmacokinetics in sparse data situations. Using only a few samples per subject the kinetics of a drug and its variability can be studied in the population of patients actually receiving the drug. Inter-individual variability may be explained by including easily measurable predictors. However, by restricting NONMEM use to this limited area, a potentially powerful statistical technique is only partially exploited.

The statistical reasons for applying mixed effects model methodology to nonlinear regression situations extend beyond the ability to combine information which may not be available in all subjects. The amount of information each individual supplies determines the weight that is given to its influence in the final population estimate. Missing measurements are not a problem in this context; they are naturally absorbed in the process. The alternative 'two-stage-method' which consists of first estimating the model parameters for each individual and subsequently combining them to obtain population information may have serious drawbacks even if adequate parameter estimates can be obtained. It assumes all individual estimates to be equally precise while they may be based on data sets with unequal numbers of measurements or missing measurements at crucial time points.

Even though further fundamental research into these analysis techniques is necessary and ongoing, this should not stop data analysts from applying what is already well developed. For some of the examples described in this paper, the alternatives do not span beyond simple averages, maximal effects or areas under the curve. This paper demonstrates the benefits that may be obtained by applying readily available software and techniques to problems that were formerly impossible to solve.

## Note

For information regarding the acquisition, installation and use of NONMEM, please contact GloboMax LLC, 7250 Parkway Drive, Suite 430, Hanover, Maryland 21076 USA; www.globomaxnm.com

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## Appendix

Reparameterising A/B in terms of clearance (Cl):
from Gibaldi and Perrier (Pharmacokinetics; second edition. Marcel Dekker Inc., New York, 1982):

$A = D \bullet (k_{21}-\alpha)/(V_c \bullet (\beta-\alpha))$ equation 2.102 page 85
$B = D \bullet (k_{21}-\beta)/(V_c \bullet (\alpha-\beta))$ equation 2.102 page 85
$k_{21} = \alpha \bullet \beta k_{10}$ equation 2.100 page 85
$Cl = V_c \bullet k_{10}$ equation 2.215 page 106

By filling in and rearanging we get:

$A/B = (\alpha \bullet \beta \bullet V_c - \alpha \bullet Cl)/(\beta \bullet Cl - \alpha \bullet \beta \bullet V_c)$

NMTRAN Code for generating the analysis resulting in Table I:

```
$PROB   2 compartment kinetics; paired
$INPUT  ID TRT AMT RATE TIME DV MDV EVID
$DATA   DATA.DAT
$SUBR   ADVAN3, TRANS5
$PK
  CL=THETA(1)*EXP(ETA(1))       ;clearance (l/min)
  TALFA=THETA(2)*EXP(ETA(2))    ;initial half-life (min)
  ALPHA=0.693/TALFA
  V=THETA(4)*EXP(ETA(4))        ;central volume of distribution (l)
  S1=V/1000                     ;corrects for different dose(mg) and conc(ng/ml) units
  TBETA = THETA(3)*EXP(ETA(3))  ;terminal half-life (min)
  BETA = 0.693/TBETA
  AOB=(V*ALPHA*BETA-CL*ALPHA)/(CL*BETA-V*ALPHA*BETA)
$ERROR
   PRDI = F                     ;Individual predicted concentrations
   Y=F*EXP(EPS(1))              ;log-normal error model
$THETA .032 94  350 4.85
$OMEGA .1 .1 .1 .1
$SIGMA .1
$EST   MAXEVAL=9999 SIGDIGITS=4 PRINT=1 NOABORT POSTHOC METHOD=1
$COV
$TABLE ID TRT TIME CL TALFA TBETA V PRDI FILE= DATA.ASC NOHEADER NOPRINT
```

The data file data.dat:

```
      1       1   1  .0500       0        .     1    4
      1       1   .  .          20     205.00   0    0
      1       1   .  .          35     157.00   0    0
...several lines removed...
      1       1   .  .        1438        .     1    2
      2       1   1  .0500       0        .     1    4
      2       1   .  .          20     222.00   0    0
      2       1   .  .          35     202.00   0    0
      2       1   .  .          83     125.00   0    0
...etc...
```

NMTRAN Code for generating the analysis resulting in table II:

```
$PROB   Clexane antiXa; Stiekema (modified) data; 2 compartments; CV Beta fixed; CLEX3
$INPUT  ID AMT TIME XA=DV MDV  EVID
$DATA   CLEXMOD.DAT
$SUBR   ADVAN3,TRANS5
$PK
  THALA = THETA(1)*EXP(ETA(1))   ;Distribution Half-life (min)
  ALPHA = 0.693/THALA
  THALB = THETA(2)*EXP(ETA(2))   ;Elimination Half-life (min)
  BETA  = 0.693/THALB
  CL    = THETA(3)*EXP(ETA(3))   ;Clearance (ml/min)
  S1    = THETA(4)*EXP(ETA(4))   ;Vc (ml) (Central volume of distribution)
  AOB   = (S1*ALPHA*BETA - CL*ALPHA)/(CL*BETA - S1*ALPHA*BETA)
                                 ;Code to allow estimation of Cl instead of AOB
$ERROR
  PRDI  = F                      ;Individual antiXa predictions
  Y     = F*EXP(EPS(1))          ;Log-normal error model
$THETA 180  2400  1.6  5000
$OMEGA .5 .1 .5 .5
$SIGMA .2
$EST   SIGDIGITS=3 PRINT=1 MAXEVAL 5000 NOABORT POSTHOC METHOD=1
$COV
$TABLE ID TIME THALA THALB AOB CL S1 PRDI FILE=CLEX3.ASC NOHEADER NOPRINT
```

The data file clexmod.dat:

```
      1  4887.00       0     .        1    1
      1      .        14     .70000   0    0
      1      .        30     .63000   0    0
...several lines removed...
      2      .      4352     .        1    2
      3  4887.00       0     .        1    1
      3      .        15     .94300   0    0
      3      .        30     .83300   0    0
      3      .        59     .66300   0    0
      3      .       120     .58300   0    0
...etc...
```

NMTRAN Code for generating the analysis resulting in table III:

```
$PROB   Clexane antiXa; additive error; unmodified data; 1 compartment; CLEX_XA1
$INPUT  ID AMT TIME XA=DV MDV  EVID
$DATA   CLEXANE.DAT
$SUBR   ADVAN1
$PK
   THAL = THETA(1)*EXP(ETA(1))      ;Elimination half-life (min)
   K    = 0.693/THAL
   CL   = THETA(2)*EXP(ETA(2))      ;Clearance (ml/min)
   S1   = CL/K
   INT  = THETA(3)*EXP(ETA(3))      ;Basal activity (IU/ml)
$ERROR
   E    = F+INT                     ;Individual antiXa predictions (with basal act.)
   Y    = E+EPS(1)                  ;Normal (additive) error model
$THETA  150  11  0.01
$OMEGA  0.1  .1  .2
$SIGMA .02
$EST   SIGDIGITS=3 PRINT=1 MAXEVAL 5000 NOABORT POSTHOC METHOD=1
$COV
$TABLE ID TIME THAL CL K S1 INT E FILE=CLEX_XA1.ASC NOHEADER NOPRINT
```

The data file clexane.dat:

```
      1  4887.00       0     .        1    1
      1      .        14     .70000   0    0
      1      .        30     .63000   0    0
...several lines removed...
      2      .      4352     .01000   0    0
      3      .         0     .01000   0    0
      3  4887.00       0     .        1    1
      3      .        15     .95000   0    0
      3      .        30     .84000   0    0
      3      .        59     .67000   0    0
      3      .       120     .59000   0    0
...etc...
```

## NMTRAN Code for generating the analysis resulting in table IV:

```
$PROB   Fragmin XA/APTT PK/PD; Free PK, exponential CE, conditional estimation; XAAPTT8
$INPUT  ID TRT DOSE=AMT TIME DV TYPE MDV EVID CMT
$DATA   XA&APTT.DAT
$SUBR   ADVAN2
$PK
  THAL  = THETA(1)*EXP(ETA(1))     ;Elimination half-life
  K     = 0.693/THAL
  CL    = THETA(2)*EXP(ETA(2))     ;Clearance
  S2    = CL/K
  TABS  = THETA(3)*EXP(ETA(3))     ;Absorption half-life
  KA    = 0.693/TABS
  F1    = THETA(4)*EXP(ETA(4))     ;Bio-availability
  INT   = THETA(5)*EXP(ETA(5))     ;Basal anti-Xa activity
$ERROR
  FXA   = F+INT                    ;Individual antiXa predictions (with basal act.)
  E0    = THETA(6)*EXP(ETA(6))     ;Basal APTT level
  I10   = THETA(&)*EXP(ETA(7))     ;antiXa increase corresponding to 10% APTT increase
  E     = E0*EXP(.09531*FXA/I10)   ;The exponential concentration-effect relationship
                                   ;providing individual APTT predictions
  Y1    = FXA+EPS(1)               ;Normal (additive) error model for antiXa measurements
  Y2    = E+EPS(2)                 ;Normal (additive) error model for APTT measurements
  Y     = (2-TYPE)*Y1+(TYPE-1)*Y2  ;If TYPE=1 then this is an antiXa measurement
                                   ;If TYPE=2 then this is an APTT measurement
$THETA  95  46  85 .78 .017  31 .07
$OMEGA .05 .05 .05  .2  .04 .02  .1
$SIGMA .0003 3
$EST   SIGDIGITS=3 PRINT=1 MAXEVAL 5000 NOABORT POSTHOC METHOD=1
$COV
$TABLE ID TRT TIME FXA E TYPE EVID THAL CL TABS F1 INT EO I10 FILE=XAAPTT8.ASC NOHEADER NOPRINT
```

## The data file xa&aptt.dat:

```
      1   1    0    0     .00500    1   0    0   2
      1   1    0    0   37.20000    2   0    0   2
      1   1  2500   0     .         1   1    1   2
      1   1    0   15     .31000    1   0    0   2
      1   1    0   15   63.20000    2   0    0   2
      1   1    0   30     .25000    1   0    0   2
      1   1    0   30   58.20000    2   0    0   2
...several lines removed...
      1   1    0 2160     .         1   1    2   2
      1   1    0 2160   35.40000    2   0    0   2
      1   2    0    0     .         1   1    3   2
      1   2    0    0     .         1   1    2   2
      1   2    0    0   36.30000    2   0    0   2
      1   2  2500   0     .         1   1    1   1
      1   2    0   60     .11000    1   0    0   2
      1   2    0   60   40.20000    2   0    0   2
      1   2    0  120     .18000    1   0    0   2
      1   2    0  120   41.40000    2   0    0   2
...several lines removed...
      1   2    0 2951     .02000    1   0    0   2
      1   2    0 2951   36.20000    2   0    0   2
      2   1    0    0     .         1   1    2   2
      2   1    0    0   33.55000    2   0    0   2
      2   1  2500   0     .         1   1    1   2
...etc...
```

Chapter 4

# Assessment of hepatic blood flow using continuous infusion of high clearance drugs

Rik C. Schoemaker, Koos Burggraaf & Adam F. Cohen
*British Journal of Clinical Pharmacology* 1998; **45**:463-469

**Summary**

*Aims* To provide methods for the translation of the concentration-time profile of highly cleared marker compounds into the underlying clearance and hepatic blood flow profile.
*Methods* Continuous infusion of indocyanine green or sorbitol was used to assess the effect of the hepatic blood flow modifiers exercise, somatostatin and octreotide. Three distinct methods are described for the translation of concentration into flow:
1. assuming successive phases of constant clearance
2. point to point estimation of clearance using estimates of concentration change
3. using a parametric description of the flow profile in combination with the differential equations describing the change in marker concentrations
*Results* The marker compound concentration profiles are adequately described using the different methods. Exercise results in a decrease in hepatic blood flow of about 80%. Somatostatin and octreotide elicit an indistinguishable hepatic blood flow decrease from 1.49 to 1.07 l $min^{-1}$. Return to baseline takes much longer for octreotide (half-life 126±104 min.) than for somatostatin (half-life 4.29±3.55 min.).
*Conclusions* Translation of concentration profiles into clearance profiles is possible making continuous assessment of hepatic blood flow feasible.

## Introduction

Drugs with high hepatic clearance depend on the rate of blood flow through the liver for their elimination from the system. As many of these drugs are on the market and many more are under development, knowledge of the rate of hepatic blood flow may be essential in the assessment or prediction of drug action. Additionally, assessment of changes in hepatic blood flow may be the focus of research on the (patho)physiology of the liver.

A number of techniques have been successfully employed to assess hepatic blood flow. This paper will focus on a kinetic approach that uses the fact that the concentration-time profile of drugs with predominant hepatic clearance provides information on hepatic blood flow [1].

Traditional kinetic analysis techniques using intravenous bolus administration must assume that the physiologic conditions and the associated pharmacokinetic model remain stationary. Therefore, bolus injections will only provide average flow estimates over the period that concentrations are measured, and timing of injections is critical when investigating short lasting changes in hepatic blood flow. On the other hand, continuous assessment of the effects of changing hepatic blood flow on plasma concentration is possible, by frequent monitoring of plasma concentrations during a zero-order infusion of a marker compound [2]. The resulting concentration profile must however still be translated into a flow profile; an abrupt change in flow for example will result in a more gradual change in concentration, governed by the half-life of the marker compound. This paper describes three closely related ways of extracting information on hepatic blood flow from concentration profiles. This is illustrated using two examples with the marker compounds indocyanine green and sorbitol, displaying different kinetic behaviour and requiring different solutions. The emphasis of this paper is on methodology and the presented examples serve as illustration only; clinical results may be reported elsewhere.

### *Kinetics during infusion*

The plasma concentration-time profile ($C_t$) for drugs that can be described by a one-compartment open model during a constant rate infusion (with rate $R_{inf}$) is given by the usual formula:

$$C_t = \frac{R_{inf}}{Cl}\left(1 - e^{\frac{Cl}{Vd}t}\right) \qquad (1a)$$

where $t$ is the time after the start of the infusion, $Cl$ is plasma clearance and $Vd$ the volume of distribution. After the infusion stops (after $T$ minutes), the decline in concentration is described by:

$$C_t = \frac{R_{inf}}{Cl}(1 - e^{-\frac{Cl}{Vd}T})e^{-\frac{Cl}{Vd}(t-T)} \qquad (1b)$$

Clearance and volume are assumed constant.
The relationship between hepatic plasma clearance and hepatic blood flow ($Q$) is given by:

$$Cl = (1 - Ht)Q \bullet E \qquad (2)$$

where $E$ is the extraction ratio and $Ht$ is the haematocrit needed to translate blood volume to plasma volume. There are a number of models describing the relationship between hepatic blood flow and extraction ratio. The two oldest, most simple and best known are the parallel-tube or undistributed sinusoidal model and the well-stirred or venous equilibration model [3,4]. More advanced models (like the dispersion, series-compartment and distributed sinusoidal models) provide predictions intermediate to the two basic models. Therefore, the predictions of the two basic models represent the two opposite extremes of the predictions of all the available models [4]. The two basic models differ significantly in their predictions for drug behaviour after oral administration and provide very different estimates for intrinsic clearance (related to enzymatic capacity in the liver) at the same extraction ratio. Nevertheless, the shape of the predicted nonlinear relationship between hepatic blood flow and extraction ratio is similar (see Figure 1) and in practice it is difficult to determine which of the models provides the most adequate description [3]. The data presented in this paper do not in any way allow discrimination between the different extraction models; this would require direct assessment of extraction ratios and actual hepatic flow. For the well-stirred model the form of this relationship is:

$$E = \frac{f_B^u Cl_{int}}{f_B^u Cl_{int} + Q} \qquad (3a)$$

and for the parallel tube model it is:

$$E = 1 - e^{-\frac{f_B^u Cl_{int}}{Q}} \qquad (3b)$$

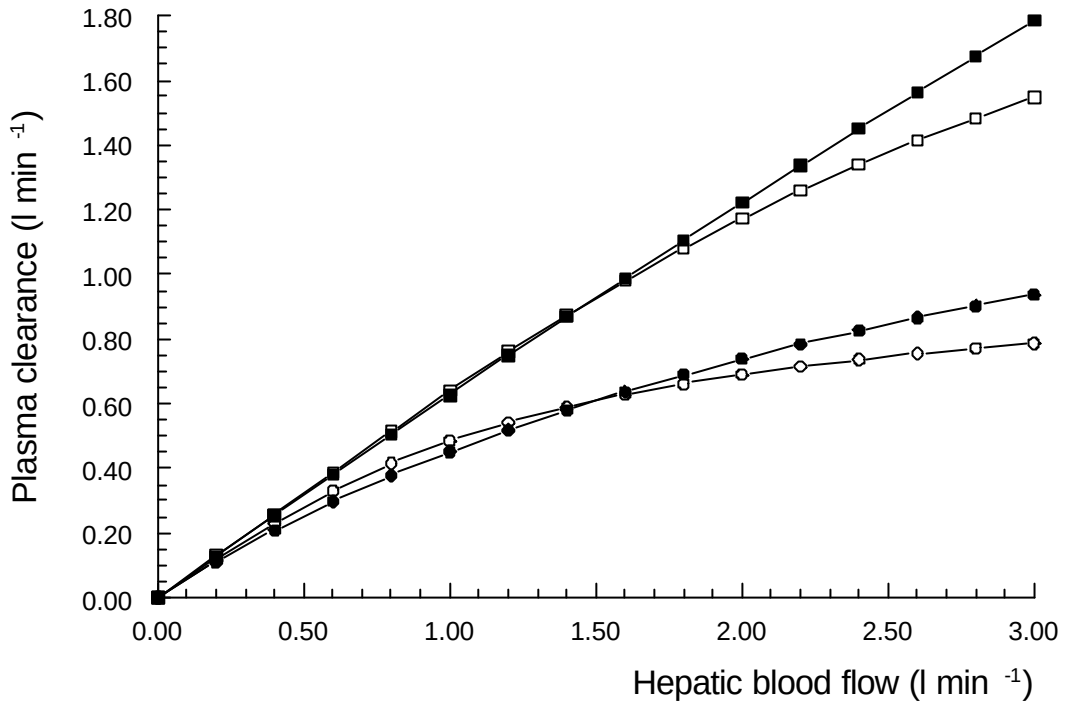


**Figure 1.** Theoretical relationship between total hepatic blood flow and plasma clearance for ICG (**Ž**/**F**) and sorbitol (•/**G**); filled markers: well-stirred model; open markers: parallel tube model

where $f_B^u$ is the fraction of unbound drug, and $Cl_{int}$ is the intrinsic clearance of the liver, related to the metabolic capacity of the liver enzymes for the cleared drug.
If the haematocrit and extraction ratio at a particular flow are known, clearance can be translated into flow. The two marker compounds that figure in this paper have different extraction ratios. The first is indocyanine green (ICG), an inert green dye used for decades in the context of hepatic blood flow estimation, with an extraction ratio of about 0.7 for healthy volunteers with a liver blood flow of around 1.5 l.min$^{-1}$ [1,5]. The second is sorbitol that has a much higher extraction ratio of around 0.96 [5]. The difference in extraction ratios results in a more linear relationship between hepatic blood flow and plasma clearance for sorbitol than for ICG (figure 1). This figure also illustrates that for flows below normal values, the relationships are virtually indistinguishable between the models.

Hepatic blood flow is seldom constant. Changes can be induced by posture, exercise, food intake, vasoactive compounds and many other circumstances [6]. This means that for a drug with a high extraction ratio, equations (1a) and (1b) are rarely applicable. The kinetics of a drug with one-compartment properties can however also be characterised using a differential equation that describes changes in concentration ($dC_t/dt$) as a function of infusion rate, drug concentration itself and the relevant kinetic parameters:

$$\frac{dC_t}{dt} = \frac{R_{inf}}{Vd} - \frac{Cl_t}{Vd} C_t \qquad (4)$$

For drugs with two-compartment pharmacokinetic properties, a system of two differential equations is required. Clearance is no longer required to be constant in these equations but may change over time ($Cl_t$). The solution to this differential equation is given by the predicted concentration-time profile. For constant clearance, this solution is provided by equations 1a and b. For clearance that changes over time, an explicit solution cannot be obtained in general and parameters have to be estimated using iterative computational techniques. However, for a limited number of clearance-time patterns an explicit solution can be obtained, as is demonstrated in the first example.

**Example 1: Hepatic blood flow during exercise**

The first example is obtained from a study in which the effect of physical exercise on hepatic blood flow was investigated during a 200 min. infusion of indocyanine green (ICG). A constant rate infusion (0.75 mg/min) of ICG was administered to eight healthy volunteers. Seventy minutes after the start of the infusion, after supposedly obtaining ICG steady state, twenty minutes of moderate physical exercise was applied. A calibrated bicycle ergometer was used, aimed at reaching a heart rate of 150 beats per minute after 5 minutes of exercise and 180 beats per minute at the end of the 20 minute exercise period. Subjects remained seated from at least 30 min prior to the start of ICG infusion until the last blood sample was taken. ICG was measured using HPLC as previously described [2] in order to avoid problems with accumulation of impurities.

Two different approaches for the translation of ICG concentration-time profiles into ICG clearance-time profiles will be described. The first approach assumes a succession of three phases with constant clearance during each phase and an instantaneous change in clearance from one phase to the next: prior to exercise, during exercise and after exercise. This is one of the rare cases where an explicit solution to differential equation (4) is possible. Equation (1a) may be generalised to the situation where a certain concentration level ($C_{t0}$) is already present when starting the infusion at $t_0$:

$$C_t = \frac{R_{inf}}{Cl}\left(1 - e^{-\frac{Cl}{Vd}(t-t_0)}\right) + C_{t_0} e^{-\frac{Cl}{Vd}(t-t0)} \qquad (5a)$$

After the infusion has stopped at time $T$, the decline is given by:

$$C_t = C_T \, e^{-\frac{Cl}{Vd}(t-T)} \qquad (5b)$$

This equation allows the description of the concentration profile associated with the three successive clearance values. Each phase has its own equation, linked to the next phase by the final calculated concentration just before the change from one clearance situation to another. This value is the starting concentration (the $C_{t0}$) for the next phase. Any nonlinear regression program that allows user-defined models may be used to obtain parameter estimates. The curves from this experiment were analysed using the NLR procedure from SPSS/PC+ V4.0.1 (SPSS, Inc., Chicago, IL). Observations were iteratively reweighted by 1/(predicted concentration)$^2$ corresponding to the assumption of a constant coefficient of variation for residual error.

The second approach allows an expression for the clearance at each time point by rewriting differential equation (4):

$$Cl_t = \frac{R_{inf} - \frac{dC_t}{dt} Vd}{C_t} \qquad (6)$$

If the volume of distribution ($Vd$) is known and constant, then clearance estimates for each time point can be obtained by filling in the corresponding estimate of the rate of concentration change ($dC_t/dt$) and the measured concentration ($C_t$) for that time point. Rate of concentration change is given by the first derivative of the concentration-time curve. For this example, the least squares straight line through three adjacent concentration-time points was used to approximate the curve. The slope of this straight line was used as an estimate of $dC_t/dt$ for the middle time point. Volume of distribution was set at the $Vd$ estimate obtained with the first approach (with the three distinct clearance phases).

*Results*

Seven subjects completed the treatment; one subject was withdrawn from the study because of signs of phlebitis at the site of ICG infusion. Average ICG concentrations (±SD) are presented in Figure 2, with the average predicted ICG plasma profile using the ›three clearance phases‹ model superimposed. Plasma clearance and volume of distribution estimates for this model are presented in Table I. Hepatic blood flow estimates were calculated for both the well-stirred and the parallel tube models, assuming an extraction ratio of 0.7 at a hepatic blood flow of 1.5 l/min (meaning $f_B^u \bullet Cl_{int}$ is 3.5 for the well-stirred model and 1.8 for the parallel tube model) and a haematocrit of 0.42 (Table I). The resulting flow estimates are different for the two models but if the results are presented as a change in flow during exercise (from prior to exercise) comparable estimates result. These estimates indicate that hepatic blood flow drops by 81%±21% (well-stirred) or 84%±20% (parallel tube). Average (" SD) point to point estimates for ICG plasma clearance calculated using the second approach are presented in Figure 3, with the average ›three clearance phases‹ estimates superimposed.

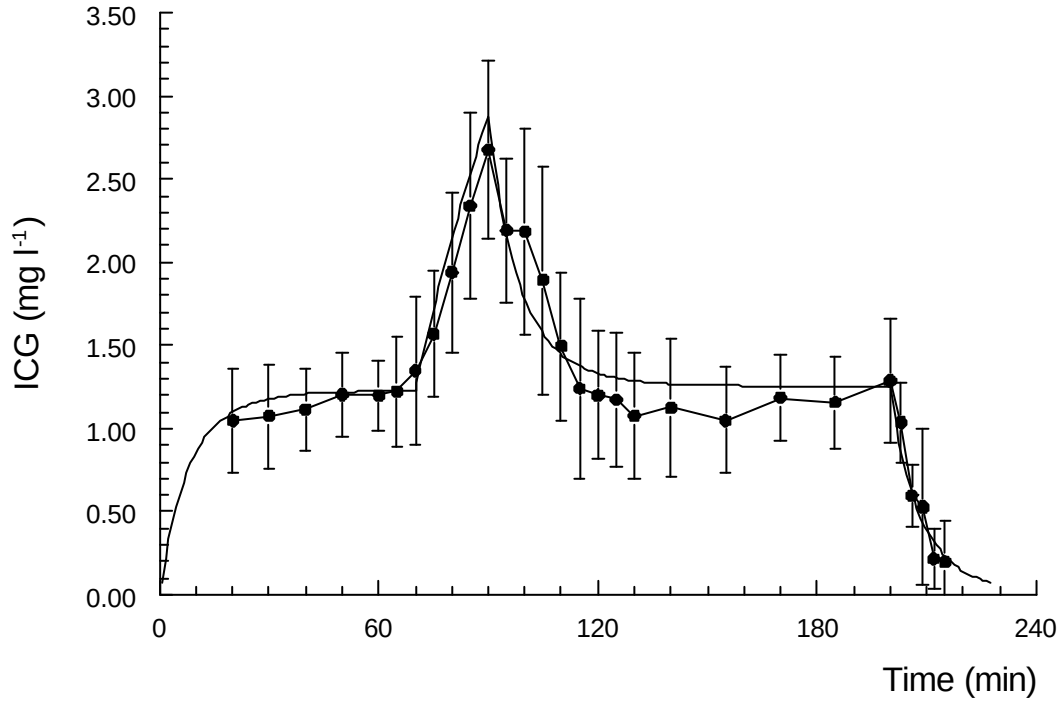


**Figure 2.** Observed (mean"SD) plasma ICG profile (**Ž**) and predicted (mean) plasma ICG profile

**Table I.** Parameters for example 1: ICG during exercise assuming three clearances phases

| | Mean | SD | Min | Max |
|---|---|---|---|---|
| Plasma clearance (l $min^{-1}$) | | | | |
| Prior to exercise | 0.632 | 0.125 | 0.455 | 0.843 |
| During exercise | 0.145 | 0.123 | 0.000 | 0.299 |
| After exercise | 0.632 | 0.158 | 0.438 | 0.890 |
| Volume of distribution (l) | 5.61 | 2.01 | 2.10 | 8.19 |
| Predicted hepatic blood flow using well-stirred model (l $min^{-1}$) (assuming E=0.7 at Q=1.5 l.$min^{-1}$) | | | | |
| Prior to exercise | 1.62 | 0.48 | 1.01 | 2.49 |
| During exercise | 0.28 | 0.25 | 0.00 | 0.60 |
| After exercise | 1.64 | 0.61 | 0.96 | 2.73 |
| Decrease due to exercise | 81% | 21% | 41% | 100% |
| Predicted hepatic blood flow using parallel tube model (l $min^{-1}$) (assuming E=0.7 at Q=1.5 l.$min^{-1}$) | | | | |
| Prior to exercise | 1.87 | 1.02 | 0.91 | 4.00 |
| During exercise | 0.25 | 0.22 | 0.00 | 0.53 |
| After exercise | 2.08 | 1.57 | 0.86 | 5.39 |
| Decrease due to exercise | 84% | 20% | 42% | 100% |

*Discussion*

There are several indications that the assumption of three constant clearances may not be correct. Figure 3 indicates that although the initial drop in clearance is rather abrupt, the return to normal values is more gradual. Also, zero clearance is estimated for two subjects during exercise, and it is unlikely that flow through the liver stops entirely during exercise, although actual extraction could drop to very low levels. Nevertheless, the constant-clearances method is appealing if interest lies in estimating average clearance during the three phases. The second approach is less restrictive because it makes no assumptions on the shape of the clearance-time profile and lends itself to the analysis of situations in which no external information is present on the time-profile of the flow-modifier. For instance, the effect of vasoactive drugs or food intake on hepatic blood flow may result in a transient change in flow, but the exact profile or the relationship between drug concentration and effect may be unknown. By translating concentrations into clearances, a point to point assessment of flow may be obtained which can be used to describe the effect profile of the intervention.

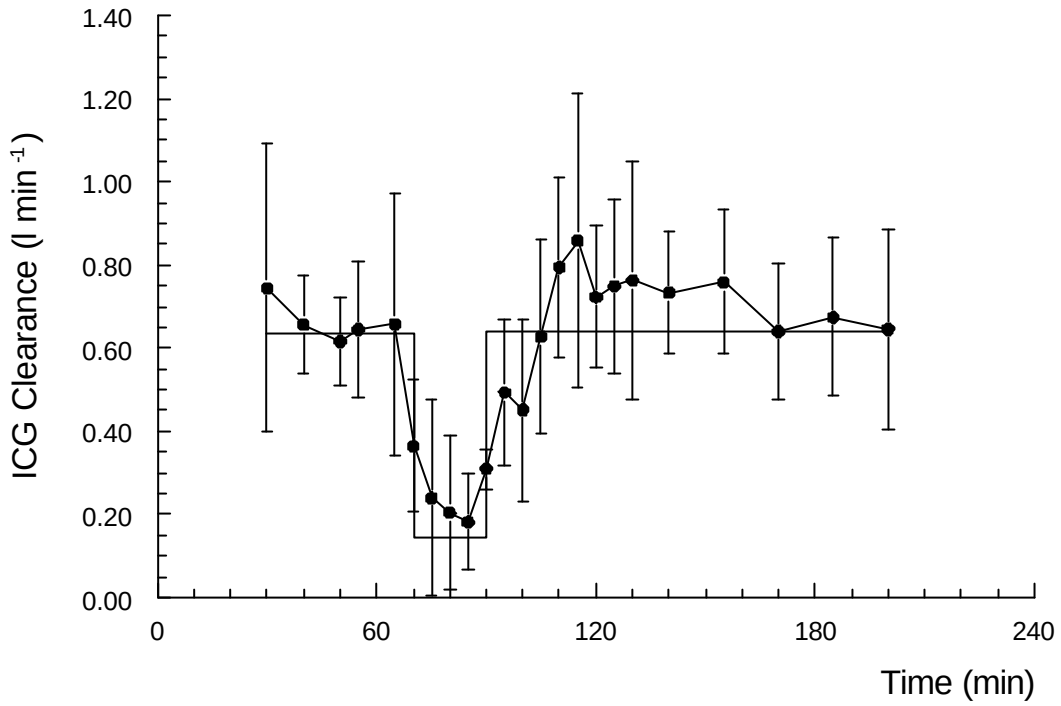


**Figure 3.** Calculated ICG plasma clearance. Predictions (mean) assuming constant clearance for the three phases (solid line) and estimates ( mean±SD) per time point (**Ž**).

Different interpolation strategies may be used to estimate the rate of concentration change. If more than three data points are used in the derivative estimation, the computed clearance profile will be smoother, but consequently abrupt changes will be blunted. Whether or not this is desirable, depends on the anticipated effect profile; an abrupt change may be unphysiological and due to measurement error. Although a simple straight line may seem like a crude approximation to the concentration-time curve, use of smoother functions like parabolas did not result in any improvement. The use of equations other than the straight line will only prove useful if the signal (true change in flow) is much larger than the noise (measurement error in marker concentration), which was not the case in this experiment.

Although ICG is reported to possess multi-compartment properties, no systematic deviations between the predictions using a one-compartment model and measured concentrations after cessation of the infusion were found. We have found no indications that more than single exponential kinetics are required to describe the concentration profile.

The choice of model determines the ultimate hepatic blood flow estimate to a sizeable extent, as does the assumed value for intrinsic clearance. If the ultimate goal is to determine the actual hepatic blood flow, then ICG may not be the best choice for a marker compound. If on the other hand, interest lies mainly in the shape of the flow profile and the fractional

changes induced, then the dependency on assumptions is minimised and ICGs more favourable properties (one-compartment kinetics, short half-life) may tip the balance.

External information on the volume of distribution is required to complete calculation of the clearance profile using the second procedure. For the data presented here, the previously obtained Vd estimate from the first approach was used. In other cases data obtained from literature may provide an approximation. The best procedure however, is probably to precede the continuous infusion by a bolus dose, accompanied by frequent sampling over a limited time span of 10 to 15 minutes. This allows accurate estimation of the volume of distribution and, as a bonus, steady state concentrations are reached sooner. For ICG infused at 0.75 mg/min, a bolus dose of 7.5 mg should suffice, provided a sufficiently sensitive assay technique is used.

**Example 2: Hepatic blood flow, somatostatin and octreotide**

The second example is from a study investigating the effect on hepatic blood flow of the drugs somatostatin and octreotide. The aim was to see if this effect could be equally measured using a continuous infusion of sorbitol as marker-compound and using echo-Doppler flow measurement in an intra-hepatic portal vein. Somatostatin is reported to induce a transient decrease in hepatic blood flow [7,8]. Octreotide is a somatostatin analogue with a much longer elimination half-life (60 *versus* 3 min) and has been shown to induce a much longer change in hepatic blood flow [9].

Treatments were administered to six healthy volunteers according to a double-blind randomised crossover design, with treatment order determined using Latin-squares balanced for first-order carry-over effects. Drugs were administered using a stepwise increasing intravenous infusion over 30 minutes to facilitate the assessment of a concentration dependant change in hepatic blood flow. The first 15 minutes, 0.6μg $min^{-1}$ of octreotide or 4μg $min^{-1}$ of somatostatin was infused, and in the remaining 15 minutes the dosing rate was doubled to 1.2μg $min^{-1}$ of octreotide or 8μg $min^{-1}$ of somatostatin. A third placebo treatment was included consisting of a 30 minute stepwise infusion with saline.

Because of the reported two-compartment pharmacokinetic properties of sorbitol, the constant rate infusion of sorbitol (0.05g per min. over 170 min.) used during hepatic blood flow assessment was preceded by a 2g loading bolus dose. A surrogate for total hepatic blood flow was determined by measuring flow in the right portal vein using echo-Doppler as described previously [10]. The right portal vein receives only a part of total hepatic flow and although strong indications exist that right portal vein flow can be used to assess relative changes in flow, absolute hepatic flow cannot be accurately determined.

Sorbitol requires a two-compartment open model to describe its kinetics. This may be implemented using the following set of differential equations:

$$\frac{dC_{1t}}{dt} = \frac{R_{inf}}{V_1} - \left( \frac{Cl_t}{V_1} + k_{12} \right) C_{1t} + k_{21} C_{2t} \qquad (7a)$$

$$\frac{dC_{2t}}{dt} = k_{12} C_{1t} - k_{21} C_{2t} \qquad (7b)$$

$C_{1t}$ and $C_{2t}$ are the concentrations in the central and peripheral compartments, $V_1$ is the volume of the central compartment and $k_{12}$ and $k_{21}$ are the micro rate constants associated with transport between the two compartments.

Solving the differential equations requires an expression for the clearance and therefore for the flow for all possible time points, and not only for the points that were actually measured. Because sorbitol requires a set of two differential equations, an essentially non-parametric expression for clearance at each time point similar to equation (6) for ICG cannot be obtained. Unfortunately, somatostatin and octreotide concentrations could not be accurately determined thereby ruling out direct (somatostatin/octreotide)concentration-(hepatic blood flow)effect modelling. Initially a model was entertained where sorbitol clearance was assumed to be a linear function of somatostatin or octreotide concentration. Since these concentrations were unavailable, a simple one-compartment pharmacokinetic model was assumed where the associated half-life was yet another parameter to be estimated. With this model, no half-life estimates for somatostatin or octreotide could be obtained leading to adequate prediction of the sorbitol profile. Inspection of the echo-Doppler results provided a clue to the explanation for this discrepance. With the previous assumptions, the decrease must be the same for each step. The Doppler data indicate however a rapid decrease in flow during the first step of the intervention and only a minor additional decrease for the second step. Additionally, the decrease in flow is similar for somatostatin and octreotide, but return to basal conditions is much slower for octreotide than for somatostatin. These two findings are in contradiction with the assumption of a linear concentration-effect relationship or the assumption of linear and one-compartment pharmacokinetics for somatostatin and octreotide, or both.

A pragmatic alternative to describe the flow-profile was required. Inspired by the shape of the Doppler flow profile, the shape of the initial drop in flow was modelled using a 30 min. constant rate infusion of substance *I* (i.e. not the two-step graded infusion of the actual intervention) to a maximum of 1 with a half-life of $t_{2\,drop}$:

$$I_t = 1 - e^{-(t - t_{start}) \frac{\ln 2}{t_{1/2drop}}} \qquad (8a)$$

To account for the distinct difference in onset and subsequent disappearance of effect, was

allowed to disappear after the end of the infusion with a different half-life ($t_{2rise}$):

$$I_t = I_{t_{stop}} \bullet e^{-(t - t_{stop})\frac{\ln 2}{t_{1/2rise}}} \qquad (8b)$$

The entire sorbitol clearance profile with $I$ equal to zero prior to the intervention was described using:

$$Cl_t = Cl_{basal} - Cl_{max} \bullet I_t \qquad (8c)$$

All sorbitol profiles for the somatostatin and octreotide occasions were analysed using the set of equations resulting from combining (7a), (7b), (8a), (8b) and (8c), resulting in estimates for parameters $V_1$, $k_{12}$, $k_{21}$, $t_{2drop}$, $t_{2rise}$, $Cl_{basal}$ and $Cl_{max}$ . Individual curves were analysed using the NONMEM software program (NONMEM Version IV, NONMEM Project Group, University of California, San Francisco, CA) which, although intended for population pharmacokinetics, can be configured to provide individual ordinary least squares estimates. An additive (constant variance) residual error model was used; syntax is presented in the appendix.

Additionally, the curves were analysed collectively using nonlinear mixed effect modelling implemented in the NONMEM software program (using first order conditional estimation). The NONMEM methodology provides estimates of mean and inter-individual variability of the population parameters, which may then be used to obtain empirical Bayes estimates for each individual and treatment. These estimates are a weighted combination of the information from the individual and the overall population information. Parameter estimates for individual curves calculated using ordinary individual least squares estimation, may depend on only one or two points and if these are ill placed (›outliers:), ridiculous estimates can result. By combining all available information from all subjects, the influence of these points is reduced, resulting in more reproducible individual estimates [11]. The method also allows formal comparison using the likelihood-ratio test of different (nested) models providing inference statements for possible differences in parameter values between treatments.

Hepatic blood flow was calculated using the predicted sorbitol clearance profile in combination with equation (2), assuming that hepatic clearance of sorbitol is 90% of total clearance [4], the extraction ratio is 0.96 (at a flow of 1.5 l.min$^{-1}$) and the haematocrit is 0.42. The well-stirred model and the parallel tube model give essentially the same translation of clearance into flow. If flow had been increased from basal values instead of decreased, this might not have been the case. The relationship between calculated hepatic blood flow and echo-Doppler measured flow in the right portal vein was investigated using conventional linear regression.

*Results*

Parameters for individual ordinary least squares descriptions of the sorbitol profile for either treatment are presented in Table IIa and IIb. These same tables present the results from the NONMEM analysis and the resulting individual empirical Bayes estimates. Using these empirical Bayes estimates, predicted profiles were generated. Sorbitol concentration measurements (mean±SD) and predictions (mean) against time are presented in figure 4. Sorbitol clearance predictions (mean±SD) and echo-Doppler flow measurements (mean±SD) against time are presented in Figure 5. Note that although the global shape of the clearance profile was inspired by the echo-Doppler data, the ultimate shape and parameter estimates are determined by the sorbitol profile alone. Parameters for the regression of predicted hepatic blood flow on echo-Doppler measured right portal vein flow are presented in Table III.

The sorbitol profile for the placebo treatment could not be described using a one-compartment model but was adequately described by a two-compartment model (Figure 4).

Several of the estimates obtained using individual ordinary least squares are clearly deviant and mean parameter values cannot generally be used due to severe outliers. This instability is greatly reduced for the empirical Bayes estimates resulting from the NONMEM analysis. The final NONMEM model presented here, estimates identical population parameters for the two treatments except for the $t_{2\,rise}$ estimate. This choice is the result of comparing different models where parameters are allowed to differ between treatments. None of the alternative model resulted in a significant improvement in fit as judged by the associated likelihood-ratio tests. This means that somatostatin and octreotide both reach the maximal effect almost immediately with a comparable decrease in clearance of 0.26±0.06 l $min^{-1}$ from 0.92±0.15 l $min^{-1}$. This translates into a lowering of hepatic blood flow from 1.48 l $min^{-1}$ to 1.07 l $min^{-1}$. Return to baseline however, is much slower for octreotide than for somatostatin. Estimated half-lives are 126±104 min and 4.29±3.55 min respectively.

**Table IIa.** Parameters for example 2: Sorbitol during infusion of octreotide

| | Mean | SD | Min | Max |
|---|---|---|---|---|
| Individual least squares estimates | | | | |
| $t_{2\,drop}$ (min) | 0.62 | 0.83 | 0.13 | 2.28 |
| $t_{2\,rise}$ (min) | 195 | 217 | 29 | 624 |
| $Cl_{basal}$ (l $min^{-1}$) | 0.930 | 0.232 | 0.696 | 1.240 |
| $Cl_{max}$ (l $min^{-1}$) | 0.283 | 0.098 | 0.130 | 0.418 |
| NONMEM estimates | | | | |
| $t_{2\,drop}$ (min) | 0.19 | 0.00 | | |
| $t_{2\,rise}$ (min) | 126 | 104 | | |
| $Cl_{basal}$ (l $min^{-1}$) | 0.920 | 0.148 | | |
| $Cl_{max}$ (l $min^{-1}$) | 0.256 | 0.063 | | |
| Empirical Bayes estimates | | | | |
| $t_{2\,drop}$ (min) | 0.19 | 0.00 | 0.19 | 0.19 |
| $t_{2\,rise}$ (min) | 137 | 85 | 64 | 295 |
| $Cl_{basal}$ (l $min^{-1}$) | 0.964 | 0.184 | 0.763 | 1.225 |
| $Cl_{max}$ (l $min^{-1}$) | 0.276 | 0.065 | 0.175 | 0.358 |

**Table IIb.** Parameters for example 2: Sorbitol during infusion of somatostatin

| | Mean | SD | Min | Max |
|---|---|---|---|---|
| Individual least squares estimates | | | | |
| $t_{2\,drop}$ (min) | 31.3 | 76.0 | 0.08 | 186.5 |
| $t_{2\,rise}$ (min) | 24.7 | 36.8 | 0.11 | 78.4 |
| $Cl_{basal}$ (l $min^{-1}$) | 0.926 | 0.174 | 0.738 | 1.213 |
| $Cl_{max}$ (l $min^{-1}$) | 0.819 | 1.302 | 0.268 | 3.476 |
| NONMEM estimates | | | | |
| $t_{2\,drop}$ (min) | 0.19 | 0.00 | | |
| $t_{2\,rise}$ (min) | 4.29 | 3.55 | | |
| $Cl_{basal}$ (l $min^{-1}$) | 0.920 | 0.148 | | |
| $Cl_{max}$ (l $min^{-1}$) | 0.256 | 0.063 | | |
| Empirical Bayes estimates | | | | |
| $t_{2\,drop}$ (min) | 0.19 | 0.00 | 0.19 | 0.19 |
| $t_{2\,rise}$ (min) | 6.37 | 6.78 | 2.24 | 20.02 |
| $Cl_{basal}$ (l $min^{-1}$) | 0.906 | 0.139 | 0.742 | 1.096 |
| $Cl_{max}$ (l $min^{-1}$) | 0.259 | 0.047 | 0.192 | 0.328 |

$t_{2\,drop}$ : half-life for flow decrease during intervention; $t_{2\,rise}$ : half-life for return to basal situation;
$Cl_{basal}$ : basal plasma clearance; $Cl_{max}$ : maximal drop in plasma clearance

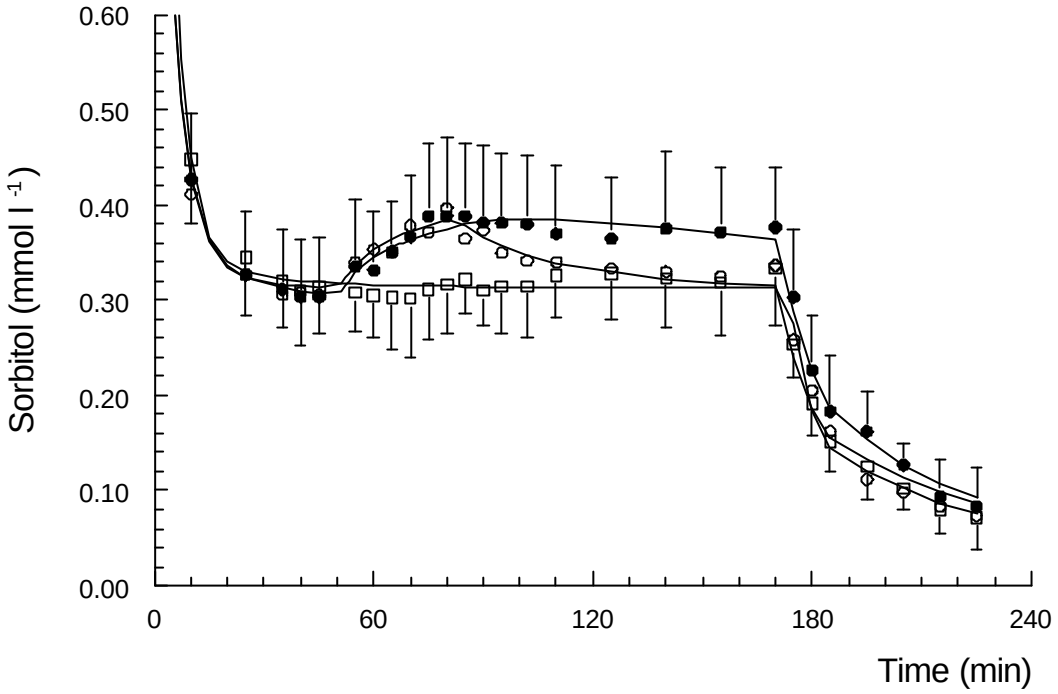


**Figure 4.** Observed (mean±SD) and predicted (mean) profiles of sorbitol plasma concentration for the octreotide (**Ž**) somatostatin (**F**) and placebo (**G**) treatment

The data provided insufficient information for the NONMEM methodology to estimate inter-individual variability in the half-life for flow-drop during the intervention ($t_{2\,drop}$). This causes the empirical Bayes estimates for all subjects to be identical. Although this seems unlikely, it simply means that the predicted profile does not improve if different values are estimated for the different individuals.

The predicted sorbitol clearance profile shows some correspondence to the measured echo-Doppler flows. Regression of predicted hepatic blood flow onto measured right portal vein flow indicates that the intercept is non-zero (0.56 ± 0.15 l $min^{-1}$). This may indicate that there is a constant amount of flow unaffected by the interventions which could be attributed to hepatic arterial flow. Slope-estimates have approximate three-fold inter-individual variability corroborating the impression that echo-Doppler measurements cannot be used to obtain a reliable estimate of absolute total hepatic blood flow. Changes in flow however, seem to be adequately reflected.

**Table III.** Parameters for example 2: Predicted hepatic blood flow *versus* measured echo-Doppler right portal vein flow

| | Mean | SD | Min | Max |
|---|---|---|---|---|
| Octreotide | | | | |
| slope | 2.06 | 0.89 | 0.89 | 3.34 |
| intercept (l $min^{-1}$) | 0.538 | 0.202 | 0.296 | 0.884 |
| | | | | |
| Somatostatin | | | | |
| slope | 2.03 | 0.85 | 0.92 | 2.98 |
| intercept (l $min^{-1}$) | 0.585 | 0.091 | 0.486 | 0.735 |

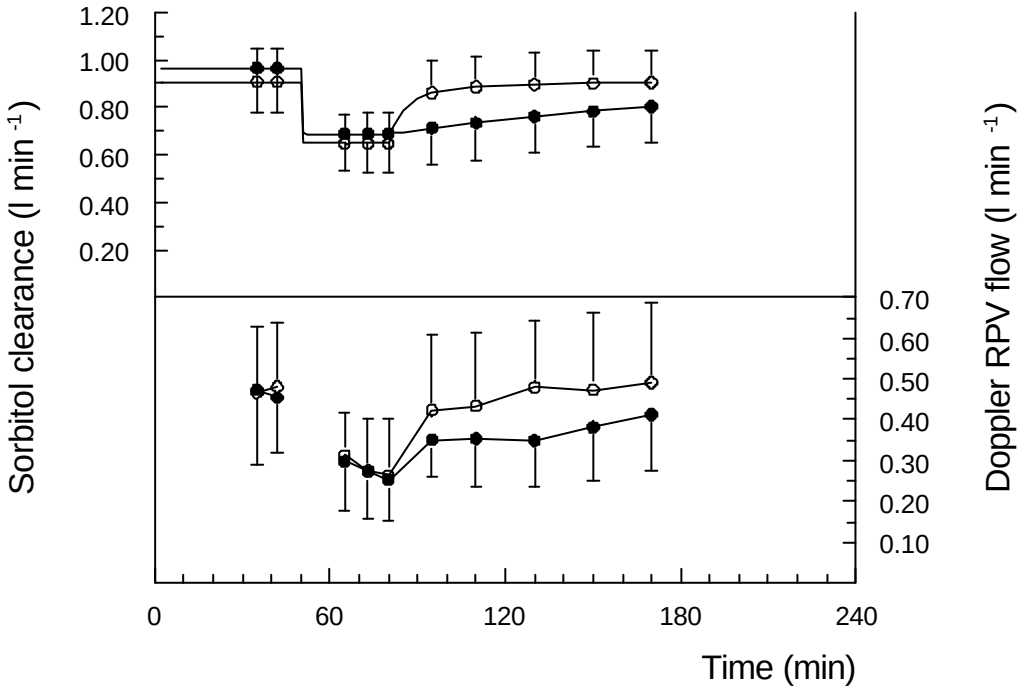


**Figure 5.** Top panel: predicted (mean±SD) profiles of sorbitol plasma clearance for the octreotide (**Ž**) and somatostatin (**F**) treatment. Bottom panel: observed (mean±SD) echo-Doppler measured flow in the right portal vein for the octreotide (**Ž**) and somatostatin (**F**) treatment

*Discussion*

The most complex part in this analysis is obtaining a continuous description of the clearance/flow profile that will result in satisfactory prediction of sorbitol concentration. Relatively simple approaches would be measuring the intervening drug and estimating the concentration effect relationship, or linear interpolation in the echo-Doppler flow profile assuming this to be a constant fraction of total hepatic flow. However, somatostatin and octreotide concentrations could not be adequately determined eliminating PK/PD modelling as an option. The first Doppler measurement during the intervention is at the end of the first step and therefore contains no information on the actual flow profile during that first step. Linear interpolation in the Doppler profile, resulted in flow-predictions changing too slowly to predict the acute rise in sorbitol concentrations immediately following the intervention. We therefore had to resort to a somewhat artificial representation, which nevertheless seems to capture the essence of the experimental results. In order to obtain an adequate sorbitol concentration profile, an immediate drop in clearance is required with gradual recovery to the basal situation. This is substantiated by the echo-Doppler measurements. The fact that the onset of effect has a much shorter half-life than the return to basal conditions is an indication of nonlinearity in either the kinetics of the intervening drugs or of the concentration-effect relationship (or both).

All modelling exercises so far have assumed that intrinsic clearance of the marker compound is unaffected by the interventions and that changes in marker concentration are attributable to changes in flow only. Echo-Doppler profiles indicate that changes in flow are actually present and for the ICG/exercise example, we may assume on the basis of physiology that flow changes do actually occur. Nevertheless, if intrinsic clearance is affected, this will influence the adequacy of the predictions for hepatic blood flow. Given the high intrinsic clearance for sorbitol, even large modifications would probably not result in major changes in extraction ratio, although this does depend on which model is considered most accurate. Halving the intrinsic clearance results in a change in extraction ratio from 0.96 to 0.92 for the well-stirred model but for the parallel tube model the extraction ratio drops to 0.80. The range of possible extraction ratios for ICG is much wider and the choice of model has greater consequences. This means that ICG cannot be used to provide reliable point estimates of hepatic blood flow without direct measurement of the extraction ratio. Description of changes in flow and their relative magnitude are far less affected though, and if interest lies here, ICG should not be dismissed as a useful marker compound.

## Conclusions

The concentration profiles that result from infusion of a high clearance drug can be translated into corresponding clearance and flow profiles. This is much easier for a drug with one-compartment kinetics like ICG than for sorbitol that requires a two-compartment model; postulation of a parametric model for the flow profile is required in this case.

The high extraction ratio for sorbitol allows easier translation from clearance to flow than for ICG, and is probably less subject to inter-individual variability. This may favour the use of sorbitol in steady state conditions for the accurate determination of hepatic blood flow. Dynamic situations, where interest lies mostly in the profile and relative magnitude of flow-changes, may favour the use of ICG.


## Acknowledgements

We would like to thank Dr. Paul Soons for the use of his ICG data.


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## Appendix

### SPSS/PC+ syntax for example 1; icg during exercise

```
get file = 'exercise.sys'.
title 'three clearance phases'.
select if (subject = 1).
compute wt=1.
weight by wt.
compute t1=0.
if (time LE 70) t1 = 1.
compute t2=0.
if ((time > 70) and (time LE 90)) t2 = 1.
compute t3=0.
if ((time > 90) and (time LE 203)) t3 = 1.
compute t4=0.
if (time GT 203)t4 = 1.
model program cl1 = 0.5 cl2 = 0.3 cl3=0.5 .
compute A  = t1*((0.75/cl1)*(1-exp(-(cl1/3.5)*time))).
compute l1 = (0.75/cl1)*(1-exp(-70*cl1/3.5)).
compute B1 = l1*exp(-(cl2/3.5)*(time-70)).
compute B2 = (0.75/cl2)*(1-exp(-(cl2/3.5)*(time-70))).
compute B  = t2*(B1 + B2).
compute l2 = l1*exp(-20*cl2/3.5) + (0.75/cl2)*(1-exp(-20*cl2/3.5)).
compute C1 = l2*exp(-(cl3/3.5)*(time-90)).
compute C2 = (0.75/cl3)*(1-exp(-(cl3/3.5)*(time-90))).
compute C  = t3*(C1+C2).
compute l3 = l2*exp(-113*cl3/3.5) + (0.75/cl3)*(1-exp(-113*cl3/3.5)).
compute D  = t4*l3*(exp(-(cl3/3.5)*(time-203))).
compute pred = A+B+C+D.
compute wt = 1/(pred*pred).
nlr icg with time t1 t2 t3 t4/save pred.
list time icg pred.
title "Instantaneous estimates".
select if (not missing(icg)).
compute x3 = time.
compute x2 = lag(time).
compute x1 = lag(x2).
compute y3 = ICG.
compute y2 = lag(ICG).
compute y1 = lag(y2).
compute sx = (x1+x2+x3).
compute sx2 = (x1*x1 + x2*x2 + x3*x3).
compute sy = (y1+y2+y3).
compute sxy = (x1*y1 + x2*y2 + x3*y3).
compute b = (sxy - (sx*sy/3))/(sx2 - (sx*sx/3)).
compute cl = (0.75 - b*3.5)/y2.
format b (F7.5) Y2 (F5.3) X2 (F3.0).
compute midtime = x2.
compute icg_ = y2.
list subject midtime icg_ b cl.
```

## Nonmem syntax for example 2; sorbitol, somatostatin and octreotide
## Individual ordinary least squares estimates

```
$PROB   93103 s alone ;rise and drop diff;one inf;2stage;2comp;S1_4
$INPUT  ID TRT AMT RATE TIME EXPT DV TYPE MDV EVID CMT PCMT
$DATA   SORBONLY.NM

$SUBROUTINES ADVAN6 TRANS1 TOL=4
$MODEL COMP=(CENTRAL,DEFDOSE,DEFOBS) COMP=(PERIPH) COMP=(FLOW)

$PK
S1  = THETA(1)*EXP(ETA(1))
K12 = THETA(2)*EXP(ETA(2))
K21 = THETA(3)*EXP(ETA(3))
CL0 = THETA(4)*EXP(ETA(4))
RISE = THETA(5)*EXP(ETA(5))
DROP = THETA(6)*EXP(ETA(6))
CLS = THETA(7)*EXP(ETA(7))
IF (TIME.LE.80) THEN
  DUM2 = 1
ELSE
  DUM2 = 0
ENDIF
HALF = DUM2*RISE + (1-DUM2)*DROP
K30 = 0.693/HALF

$ERROR
  SORI = F
  Y = SORI+EXP(ETA(8))*ERR(1)

$DES
  DADT(3)=-K30*A(3)
  DIV3 = 0.666/(0.693/RISE)
  C3 = A(3)/DIV3
  CL = CL0 - CLS*C3
  K=CL/S1
  DADT(1)=K21*A(2) - (K12+K)*A(1)
  DADT(2)=K12*A(1) - K21*A(2)

$THETA  (1,11,20) (0.0001,.071,1) (0.0001,.066,1) 0.9  (0.01,1)
        (0.1,8) (0.0001,0.3)
$OMEGA   100 100 100 100 100 100 100 100
$SIGMA   1 FIXED
$EST  SIGDIG=2 MAXEVAL=0 NOABORT POSTHOC METHOD=1 INTERACTION
$TABLE ID TRT TIME EXPT S1 K12 K21 CL0 RISE DROP CLS CL SORI
DV TYPE FILE=S1_4.ASC NOHEADER NOPRINT
```

## NONMEM syntax for example 2; first order conditional estimation

```
$PROB   93103 s alone ;rise and drop diff;one inf;CE;2comp;S1_6
$INPUT  ID TRT AMT RATE TIME EXPT DV TYPE MDV EVID CMT PCMT
$DATA   SORBONLY.NM

$SUBROUTINES ADVAN6 TRANS1 TOL=3
$MODEL COMP=(CENTRAL,DEFDOSE,DEFOBS) COMP=(PERIPH) COMP=(FLOW)

$PK
S1  = THETA(1)*EXP(ETA(1))
K12 = THETA(2)*EXP(ETA(2))
K21 = THETA(3)*EXP(ETA(3))
CL0 = THETA(4)*EXP(ETA(4))
RISE = THETA(5)*EXP(ETA(5))
DROP1 = THETA(6)
DROP2 = THETA(8)
DRP = (2-TRT)*DROP1+(TRT-1)*DROP2
DROP = DRP*EXP(ETA(6))
CLS = THETA(7)*EXP(ETA(7))
IF (TIME.LE.80) THEN
  DUM2 = 1
ELSE
  DUM2 = 0
ENDIF
HALF = DUM2*RISE + (1-DUM2)*DROP
K30 = 0.693/HALF

$ERROR
  SORI = F
  Y = SORI+ERR(1)

$DES
  DADT(3)=-K30*A(3)
  DIV3 = 0.666/(0.693/RISE)
  C3 = A(3)/DIV3
  CL = CL0 - CLS*C3
  K=CL/S1
  DADT(1)=K21*A(2) - (K12+K)*A(1)
  DADT(2)=K12*A(1) - K21*A(2)

$THETA  (1,11,20) (0.0001,.09,1) (0.0001,.12,1) 0.9  (0.01,.2)
        (0.1,30) (0.0001,0.3) (1,200,1000)
$OMEGA   .01 .01 .05 .05 .1 1 .1
$SIGMA   .0005
$EST  SIGDIG=3 PRINT=1 MAXEVAL=9999 NOABORT POSTHOC METHOD=1
$COV
$TABLE ID TRT TIME EXPT S1 K12 K21 CL0 RISE DROP CLS CL SORI
DV TYPE FILE=S1_6.ASC NOHEADER NOPRINT
```

## sorbonly.nm:

```
     1       1 10.9800 10.9800       0       0 .        2   1    4   1    1
     1       1 46.6650   .2745       1       1 .        2   1    1   1    1
     1       1   .       .           2       2 .        2   1    2   1    1
     1       1   .       .           7       7 .        2   1    2   1    1
     1       1   .       .          10      10 .5000    2   0    0   1    1
     1       1   .       .          15      15 .        2   1    2   1    1
     1       1   .       .          20      20 .        2   1    2   1    1
     1       1   .       .          25      25 .4000    2   0    0   1    1
     1       1   .       .          35      35 .3900    2   0    0   1    1
     1       1   .       .          40      40 .3700    2   0    0   1    1
     1       1   .       .          45      45 .3600    2   0    0   1    1
```

```
1       1 10.0000   .6666      50      50 .        1   1    1   3    3
1       1   .       .          51      51 .        2   1    2   1    1
1       1   .       .          51      51 .        1   1    2   3    3
1       1   .       .          52      52 .        2   1    2   1    1
1       1   .       .          52      52 .        1   1    2   3    3
1       1   .       .          53      53 .        2   1    2   1    1
1       1   .       .          53      53 .        1   1    2   3    3
1       1   .       .          55      55 .4000    2   0    0   1    1
1       1   .       .          55      55 .        1   1    2   3    3
1       1   .       .          60      60 .4100    2   0    0   1    1
1       1   .       .          60      60 .        1   1    2   3    3
1       1   .       .          65      65 .1685    1   1    2   3    3
1       1   .       .          65      65 .4300    2   0    0   1    1
1       1 10.0000   .6666      65      65 .        1   1    1   3    3
1       1   .       .          66      66 .        2   1    2   1    1
1       1   .       .          66      66 .        1   1    2   3    3
1       1   .       .          67      67 .        2   1    2   1    1
1       1   .       .          67      67 .        1   1    2   3    3
1       1   .       .          68      68 .        2   1    2   1    1
1       1   .       .          68      68 .        1   1    2   3    3
1       1   .       .          70      70 .4600    2   0    0   1    1
1       1   .       .          73      73 .1380    1   1    2   3    3
1       1   .       .          75      75 .4800    2   0    0   1    1
1       1   .       .          80      80 .1315    1   1    2   3    3
1       1   .       .          80      80 .4700    2   0    0   1    1
1       1   .       .          82      82 .        1   1    2   3    3
1       1   .       .          85      85 .4800    2   0    0   1    1
1       1   .       .          85      85 .        1   1    2   3    3
1       1   .       .          90      90 .4900    2   0    0   1    1
1       1   .       .          90      90 .        1   1    2   3    3
1       1   .       .          95      95 .2155    1   1    2   3    3
1       1   .       .          95      95 .4600    2   0    0   1    1
1       1   .       .         102     102 .4700    2   0    0   1    1
1       1   .       .         110     110 .2238    1   1    2   3    3
1       1   .       .         110     110 .4600    2   0    0   1    1
1       1   .       .         125     125 .4600    2   0    0   1    1
1       1   .       .         132     130 .2075    1   1    2   3    3
1       1   .       .         140     140 .4700    2   0    0   1    1
1       1   .       .         150     150 .1970    1   1    2   3    3
1       1   .       .         155     155 .4700    2   0    0   1    1
1       1   .       .         170     170 .2543    1   1    2   3    3
1       1   .       .         170     170 .4600    2   0    0   1    1
1       1   .       .         175     175 .4100    2   0    0   1    1
1       1   .       .         180     180 .3100    2   0    0   1    1
1       1   .       .         185     185 .2800    2   0    0   1    1
1       1   .       .         195     195 .1800    2   0    0   1    1
1       1   .       .         208     205 .1400    2   0    0   1    1
1       1   .       .         215     215 .1200    2   0    0   1    1
1       1   .       .         220     225 .1000    2   0    0   1    1
2       1 10.9800 10.9800       0       0 .        2   1    4   1    1
2       1 46.6650   .2745       1       1 .        2   1    1   1    1
2       1   .       .           2       2 .        2   1    2   1    1
2       1   .       .           2       2 .        1   1    2   3    3
2       1   .       .           7       7 .        2   1    2   1    1
```

...etc..

Chapter 5

# Estimating potency for the $E_{max}$-model without attaining maximal effects

Rik C. Schoemaker, Joop M.A. van Gerven & Adam F. Cohen
*Journal of Pharmacokinetics and Biopharmaceutics* 1998; **26** (5)

**Summary**

The most widely applied model relating drug-concentrations to effects is the $E_{max}$ model. In practice, concentration-effect relationships often deviate from a simple linear relationship but without reaching a clear maximum because a further increase in concentration might be associated with unacceptable or distorting side-effects. The parameters for the $E_{max}$ model can only be estimated with reasonable precision if the curve shows sign of reaching a maximum, otherwise both $EC_{50}$ and $E_{max}$ estimates may be extremely imprecise.

This paper provides a solution by introducing a new parameter ($S_0$) equal to $E_{max}/EC_{50}$ that can be used to characterise potency adequately even if there are no signs of a clear maximum. Simulations are presented to investigate the nature of the new parameter and published examples are used as illustration.

## Introduction

The relationship between concentration and effect for a drug can often be characterised using relatively simple forms. In practice almost all applications make use of one of three models which, with increasing complexity, are the linear, the $E_{max}$ and the sigmoid $E_{max}$ model. It could be argued that the $E_{max}$ model is central, with the sigmoid $E_{max}$ model as an empirical extension allowing extra flexibility, and the linear model as an approximation to a part of the $E_{max}$ model. A particular $E_{max}$ model equation where the drug leads to a decrease in response from the basal situation is given by:

$$E_i = E_0 - \frac{E_{max} C_i}{EC_{50} + C_i} \qquad (1)$$

where $E_i$, the effect at concentration $C_i$, is given as the effect in absence of drug ($E_0$) with a maximum change ($E_{max}$) and a concentration at which half the maximum effect occurs ($EC_{50}$). Naturally, by changing the minus sign in equation 1 into a plus sign, the curve will describe an increase in response with increasing drug concentrations. The left panel of Figure 1 provides an illustration of the $E_{max}$ model where $E_0$ is the intercept with the y-axis, the vertical dashed line indicates the $EC_{50}$ and the horizontal dashed line indicates the minimal obtainable effect given by $E_0$ - $E_{max}$.

The $E_{max}$ model also known as the Michaelis-Menten model (where $E_{max}$ is replaced by $V_{max}$ and $EC_{50}$ by $K_m$) originates in classical receptor theory as the model that describes binding of drug to a receptor based on the law of mass action [1]. Much of the popularity of the $E_{max}$ model stems from the analogy in form with this drug-receptor model, and from its empirical usefulness [2]. While *in-vitro* pharmacology often allows characterisation of maximum effects, this may not be obtainable in *in-vivo* experiments. *In-vivo* pharmacodynamics is often far more complex than the result of a simple interaction between the drug and a receptor. Several receptor types may be involved, there may be intermediary steps between receptor and final effect, and regulatory and counter-regulatory mechanisms may interfere. One of the results may be that the high drug levels associated with maximal effects also incur unacceptable side effects making it impossible to reach these concentrations. If, for instance, sedation due to a benzodiazepine is measured using saccadic eye movements, the subjects will generally fall asleep -making further effect measurement impossible- far before a maximum is reached on the concentration-effect curve [3,4].

Sensible parameter estimates for the $E_{max}$ model can only be obtained if the concentration-effect relationship has clear indications of levelling off and reaching a maximum. If this is not the case then both $E_{max}$ and $EC_{50}$ are highly correlated and an extreme range of values may be used to describe the same empirical relationship. This has been previously discussed in the context of pharmacokinetic models where the elimination rate is not constant, but is assumed to follow a Michaelis-Menten type relationship [5,6,7]. It is

argued that if concentrations remain well below $K_m$, a linear approximation is adequate and only the ratio of $V_{max}$ over $K_m$ (equal to the slope of this linear approximation) can be identified.

When estimating concentration-effect relationships, sometimes straight lines may suffice to describe the concentration-effect relationship, but often there are indications of deviation from linearity. If a straight line were forced through these data, the slope of the line would depend on the amount of curvature present. This is illustrated in the middle panel of Figure 1.

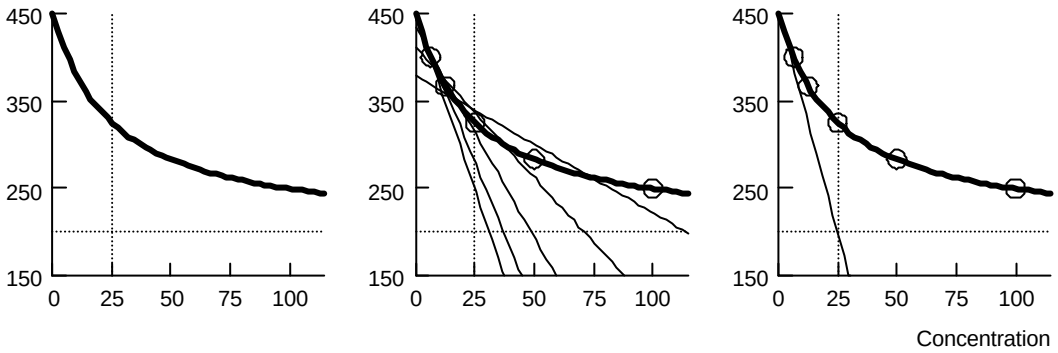


**Figure 1.** For explanation see text. Left panel: an example of an $E_{max}$ concentration-effect relationship. Middle panel: ordinary least squares straight lines for the $E_{max}$ curve truncated at the open circles. Right panel: tangent to the $E_{max}$ curve at zero concentration.

The circles on the curve correspond to the effect at concentrations equal to 25%, 50%, 100%, 200% and 400% of $EC_{50}$. If the curve were to stop at these points and straight lines would be fitted to these sections, the five increasingly more horizontal lines would result. These lines indicate that a linear approximation cannot be used unless all subjects reach the same fraction of $E_{max}$, which is unlikely in practice.

The initial part of the $E_{max}$ curve does not change with increasing concentrations and the tangent to the concentration-effect curve at zero concentration does not depend on the rest of the curve. This tangent is illustrated in the right panel of Figure 1 and is mathematically given by [7,8]:

$$E_i = E_0 - \frac{E_{\max}}{EC_{50}} C_i \qquad (2)$$

When the $E_{max}$ model is used to estimate a curve without a clear maximum, the curious situation arises that $E_{max}$ and $EC_{50}$ estimates are extremely variable while their ratio is far less so. This is because while $E_{max}$ and $EC_{50}$ are badly defined, the initial tangent is not and the ratio defines the slope of this tangent. If instead of estimating $EC_{50}$, a new parameter equal to the ratio of $E_{max}$ over $EC_{50}$ is estimated, then a more stable parameter is obtained that can be interpreted as the initial sensitivity to the drug at low concentrations. This new

parameter which we propose to call $S_0$ (the slope of the tangent at concentration zero) may be compared between drugs and between different doses of the same drug without the necessity of reaching a clear maximum. It is implemented by replacing $EC_{50}$ in equation 1 by $E_{max}/S_0$ resulting in:

$$E_i = E_0 - \frac{S_0 E_{max} C_i}{E_{max} + S_0 C_i} \qquad (3)$$

The usefulness of this alternate parameterisation is demonstrated by a number of simulations and practical examples from first-entry into man studies of two new benzodiazepines.

## Simulations

The simulations were performed to examine the behaviour of parameters of the $E_{max}$ model. The structure of the data, the kinetic and dynamic parts, and the parameters, are derived from the practical application discussed in the second part of this paper.

Time points were assumed with both concentration and effect measurements at 0, 3, 8, 13, 18, 24, 28, 33, 43, 58, 73, 88, 103, 118, 148, 178, 238 and 298 minutes after starting drug administration. For the $E_{max}$ model, parameters were used describing the peak velocity of saccadic eye movements, an effect measure for sedation [9]. The saccadic peak velocity is the maximal rate at which the eyes are capable of following a random stimulus jumping from one side of the visual field to the other. The typical response to a sedative is a decrease in saccadic peak velocity [10]. $E_0$ was set at 450 E/sec, $E_{max}$ at 250 E/sec and $EC_{50}$ at 25 ng/ml. By varying the administered dose, 5 different maximum concentrations were reached corresponding to 25%, 50%, 100%, 200% and 400% of the $EC_{50}$, which in turn correspond to 20%, 33%, 50%, 66% and 80% of $E_{max}$. For the simulations, the kinetic and dynamic parameters were kept constant, and effect measurements were simulated. The simulated data sets consisted of 100 replications (occasions) for each $C_{max}$-level.

**Table I.** Conditions for the different simulations

| Set code | Simulation conditions | | |
|---|---|---|---|
| | Residual variability | Infusion/bolus | Effect compartment |
| HR0 | high ($\sigma^2$=750) | infusion (20min) | no effect compartment |
| HB0 | high ($\sigma^2$=750) | bolus injection | no effect compartment |
| HR4 | high ($\sigma^2$=750) | infusion (20min) | effect compartment ($t_{\frac{1}{2}Keo}$=4min) |
| HB4 | high ($\sigma^2$=750) | bolus injection | effect compartment ($t_{\frac{1}{2}Keo}$=4min) |
| LR0 | low ($\sigma^2$=75) | infusion (20min) | no effect compartment |
| LB0 | low ($\sigma^2$=75) | bolus injection | no effect compartment |
| LR4 | low ($\sigma^2$=75) | infusion (20min) | effect compartment ($t_{\frac{1}{2}Keo}$=4min) |
| LB4 | low ($\sigma^2$=75) | bolus injection | effect compartment ($t_{\frac{1}{2}Keo}$=4min) |

From these data sets, the dynamic parameters were estimated while the concentration profile was assumed known. Three factors with two levels each were examined yielding the eight combinations summarised in Table I; presence versus absence of a hypothetical effect compartment [2], high versus low residual variability, and an intravenous (IV) bolus versus a constant rate infusion. The effect compartment -introduced to account for hysteresis- was simulated with an equilibration half-life ($t_{\frac{1}{2}Keo}$) of 4 minutes while equilibration half-life was a parameter to be estimated. Two different levels of intra-individual, additive normal residual variability were investigated; a variance of 750 corresponding roughly to actual saccadic peak velocity measurements (as found in the examples described later) and a ten-fold lower variance of 75. Two different infusion regimes were investigated, an IV bolus and a constant rate infusion over 20 minutes.

Data were simulated and subsequently analysed using NONMEM version IV software (NONMEM Project Group, UCSF, CA). Although NONMEM is generally used for population analyses, it can also be configured to provide ordinary individual nonlinear regression analyses [11]. Coefficients of variation of the 100 estimates obtained under the various conditions are calculated using the formula CV = %($10^{s_5 \ln 10}$ - 1) where $s_5$ is the variance of the $^{10}$log-transformed estimates [12]. Bias (systematic deviation in the mean of the estimates) is calculated by back-transforming the difference between the mean of the log-transformed estimates and the simulated value. Distribution of estimates is presented using box-whisker graphs; the boxes encompass 50 % of the estimates, the horizontal line in the middle of each box is the median and the whiskers indicate the 5th and 95th percentile of the estimates. Both untransformed and log-transformed estimates are shown. The scale of the y-axes for the log-data has the same increase from low to high (a factor $10^4$) for all parameters and therefore the size of the boxes can be compared between parameters and is proportional to the coefficient of variation.

**Table II**. Coefficients of variation and bias for estimated parameters; data are reported as coefficient of variation(%)/bias (% deviation of log mean from simulated values). Set codes indicating the different conditions for the simulations are explained in Table I. $C_{max}$ is given in percentage of $EC_{50}$.

| Set code | $C_{max}$ | $S_0$ | $EC_{50}$ | $E_{max}$ | $t_{1/2Keo}$ |
|---|---|---|---|---|---|
| HR0 | 25% | 302 / 82 | 1997 / -39 | 339 / 12 | |
| | 50% | 96 / 54 | 707 / -10 | 236 / 39 | |
| | 100% | 72 / 13 | 309 / 26 | 134 / 43 | |
| | 200% | 57 / 4 | 129 / 18 | 59 / 22 | |
| | 400% | 47 / -1 | 65 / 5 | 25 / 4 | |
| HB0 | 25% | 316 / 104 | 1876 / -46 | 317 / 10 | |
| | 50% | 116 / 45 | 815 / 7 | 230 / 55 | |
| | 100% | 73 / 5 | 372 / 64 | 162 / 72 | |
| | 200% | 73 / 20 | 156 / -1 | 63 / 19 | |
| | 400% | 68 / -1 | 84 / 9 | 20 / 8 | |
| HR4 | 25% | 270 / 103 | 1734 / -32 | 295 / 39 | 517 / -4 |
| | 50% | 76 / 41 | 733 / 66 | 297 / 134 | 156 / -23 |
| | 100% | 76 / 4 | 434 / 103 | 170 / 111 | 93 / -9 |
| | 200% | 55 / -5 | 157 / 46 | 79 / 38 | 62 / -1 |
| | 400% | 57 / 1 | 80 / 10 | 26 / 10 | 55 / -3 |
| HB4 | 25% | 423 / 223 | 2013 / -45 | 342 / 79 | 789 / 75 |
| | 50% | 184 / 71 | 1336 / 6 | 285 / 81 | 198 / -20 |
| | 100% | 86 / 15 | 593 / 160 | 223 / 199 | 120 / -10 |
| | 200% | 73 / 12 | 453 / 77 | 197 / 98 | 114 / -20 |
| | 400% | 61 / -7 | 245 / 84 | 120 / 71 | 97 / -16 |
| LR0 | 25% | 41 / 10 | 382 / 43 | 232 / 58 | |
| | 50% | 30 / 3 | 215 / 36 | 146 / 40 | |
| | 100% | 22 / -2 | 52 / 13 | 31 / 11 | |
| | 200% | 18 / 2 | 24 / 0 | 8 / 2 | |
| | 400% | 15 / -1 | 17 / 2 | 4 / 0 | |
| LB0 | 25% | 49 / 19 | 444 / 10 | 235 / 30 | |
| | 50% | 35 / 8 | 268 / 25 | 173 / 35 | |
| | 100% | 28 / -5 | 95 / 26 | 62 / 21 | |
| | 200% | 17 / -1 | 24 / 2 | 10 / 1 | |
| | 400% | 20 / -3 | 21 / 3 | 4 / 0 | |
| LR4 | 25% | 42 / 11 | 489 / 107 | 289 / 130 | 73 / -12 |
| | 50% | 31 / -2 | 340 / 112 | 227 / 108 | 29 / -2 |
| | 100% | 22 / -4 | 62 / 18 | 39 / 13 | 20 / -1 |
| | 200% | 18 / -1 | 26 / 3 | 10 / 2 | 14 / -1 |
| | 400% | 16 / -1 | 18 / 2 | 5 / 1 | 15 / 2 |
| LB4 | 25% | 116 / 54 | 1401 / -33 | 401 / 4 | 114 / -21 |
| | 50% | 44 / 15 | 481 / 65 | 280 / 89 | 48 / -10 |
| | 100% | 29 / -2 | 320 / 101 | 219 / 96 | 24 / -5 |
| | 200% | 23 / 1 | 63 / 7 | 29 / 8 | 18 / -1 |
| | 400% | 19 / -1 | 31 / 4 | 13 / 3 | 20 / 2 |

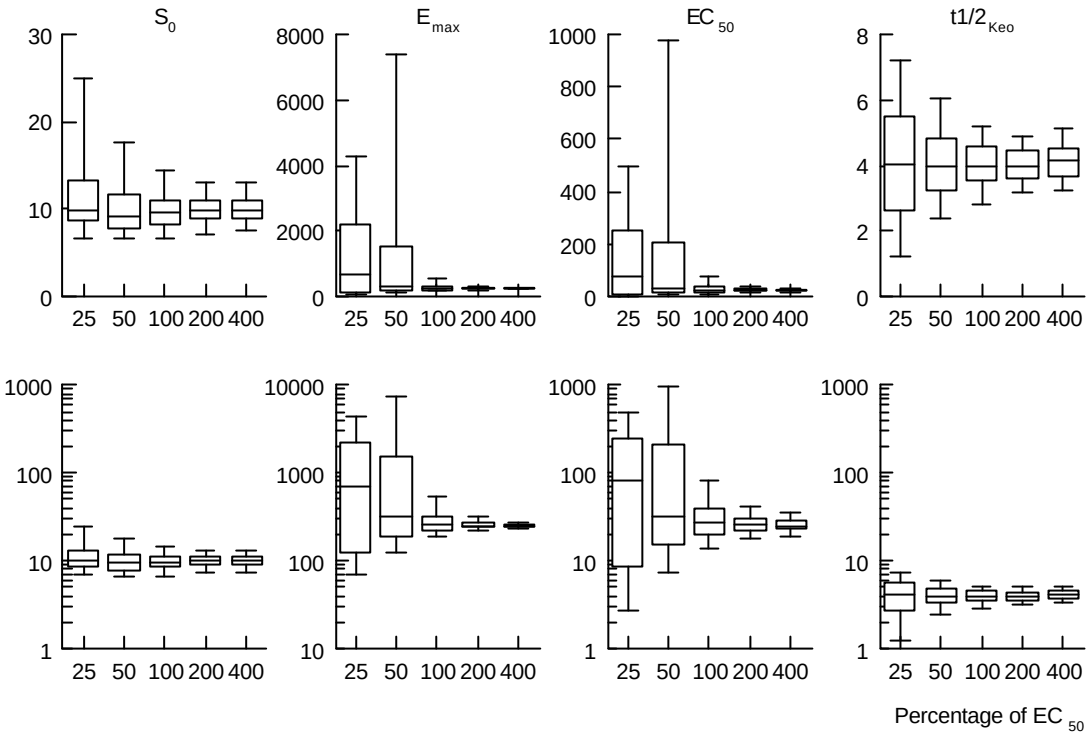


**Figure 2.** Box-whisker plots for the different parameters estimated for one of the simulations (LR4; $S_0$=10 °.sec$^{-1}$.ng$^{-1}$.ml, $E_{max}$=250 °.sec$^{-1}$, $EC_{50}$=25 ng.ml$^{-1}$, $t_{\frac{1}{2}Keo}$=4 min., $\sigma^2$=75). Values depicted are the 5th, 25th, 50th, 75th and 95th percentiles of the estimates. Top panels: untransformed estimates, bottom panels: log-transformed estimates.

*Simulations: results*

Table II lists the coefficients of variation and the bias-estimates for the eight simulations. Figure 2 gives box-whisker graphs for the estimates obtained after analysing the data set with an effect compartment, residual variability of 75 and a 20 min. infusion (LR4). The other seven simulations displayed comparable qualitative behaviour.

The graphs indicate that all untransformed parameters are extremely skewed while log-transformation makes the distributions much more symmetric. Graphs and Table II also indicate clearly that the variability of the estimate is much lower for the $S_0$ parameter than for the $EC_{50}$, especially at the lower concentration range for which it was designed. Regarding variability, $E_{max}$ takes in a position between $S_0$ and $EC_{50}$. The variability of the $E_{max}$ parameter decreases clearly with further development of the $E_{max}$ curve because more information becomes available. CVs for $S_0$ decrease in a far more gradual manner. A positive bias (systematic departure from the simulated values) in the $S_0$ and $E_{max}$ estimates is apparent from Table II. For $S_0$, the size of the bias is clearly associated with the size of the CVs and is virtually absent if $C_{max}$ is larger than the $EC_{50}$. Bias is generally less for $S_0$ than for $EC_{50}$ and $E_{max}$, except perhaps for the low (25%) $C_{max}$ conditions. Data for $E_0$ are not

presented but are characterised by symmetric distributions without the need for (log) transformation, absence of bias and constant variability over the range of $C_{max}$-values.

## Application

In recent studies two new benzodiazepines (Ro 48-6791 and Ro 48-8684) were compared to midazolam with respect to sedative effects measured using saccadic eye movements [3,4]. Both studies were first-administration-to-man, placebo-controlled rising-dose studies with two panels of five subjects each. The subjects received five treatments; placebo, midazolam and three intended doses of the new drug: panel A1 Ro 48-8684 0.1mg, 0.3mg and 1.0mg; panel A2 Ro 48-8684 1mg, 3mg and 10mg; panel B1 Ro 48-6791 0.1mg, 0.3mg and 1.0mg; panel B2 Ro 48-6791 1mg, 2mg and 3mg. The drugs were infused slowly over 20 minutes or until the subjects became too sedated to perform the saccadic eye movement test.

PK/PD information was obtained for all doses with the aim of answering two questions: 1. is the sensitivity to the new drug different from midazolam and 2. does the sensitivity change with increasing doses of the same drug. Saccadic eye movement tests cannot be done beyond the level of conscious sedation and this level is often approached with a close-to-linear concentration-effect relationship. Comparison of sensitivity between midazolam and the highest administered doses of the two new benzodiazepines using traditional methods may therefore be hampered by a nonlinear concentration-effect curve without reaching maximal effect. A change of sensitivity with increasing doses poses the problem of a slowly developing $E_{max}$ curve as illustrated in the middle panel of Figure 1.

### *Application: methods*

Concentration-effect parameters were determined using the described model ($E_0$, $E_{max}$, $S_0$, $t_{2\,Keo}$). Additive intra-individual residual error and an effect compartment were assumed. Data were analysed with NONMEM version IV using ordinary nonlinear regression analysis [11]. Parameters are presented as geometric means (back-transformed log-means) and coefficients of variation of the estimates. For the new benzodiazepines the three highest doses were compared, and the highest administered dose was compared between the different drugs. Parameters were analysed after log-transformation using unpaired Student-s t-tests for unequal variances.

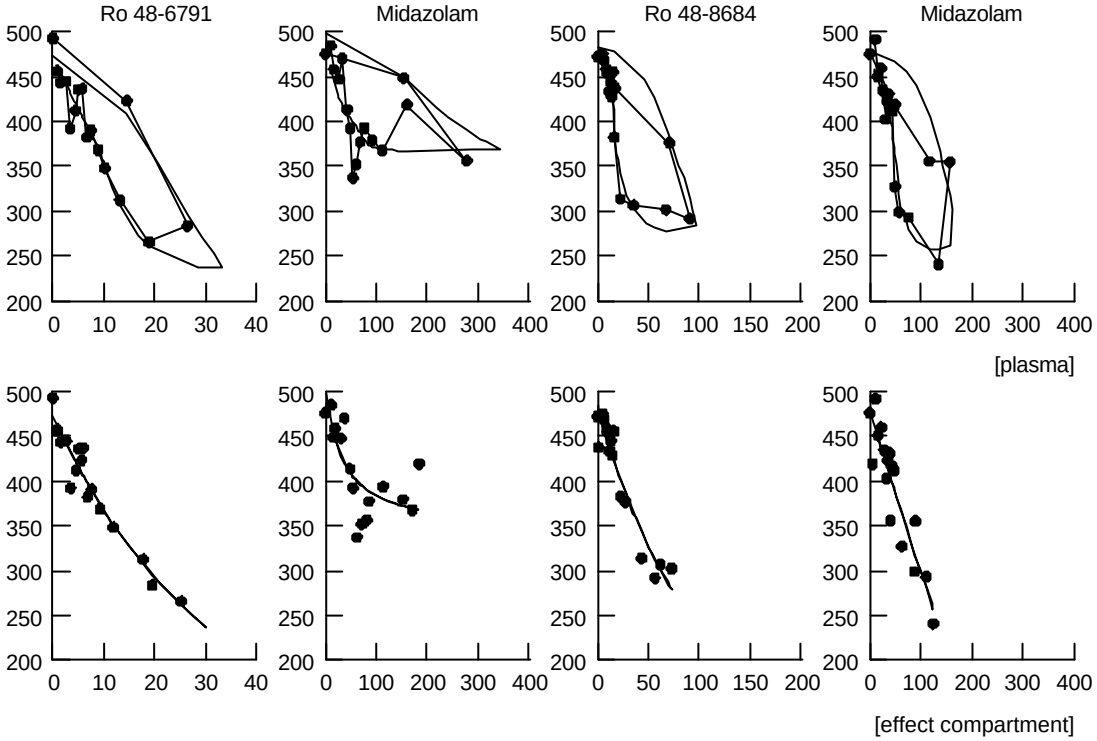


**Figure 3.** Illustrative concentration-effect graphs. Top panels: plasma concentration, bottom panels: effect compartment concentration. New benzodiazepine graphs are accompanied by the midazolam graphs for the same subject. Note the difference in scale on the x-axes.

**Table III.** Parameter estimates for the rising dose studies.
Data are reported as: geometric mean (coefficient of variation).

| Dose/Drug | $S_0$ ($°.sec^{-1}.ng^{-1}.ml$) | $E_{max}$ ($°.sec^{-1}$) | $EC_{50}$ ($ng.ml^{-1}$) | $t_{\frac{1}{2}Keo}$ (min) | n |
|---|---|---|---|---|---|
| 1mg Ro48-6791 | 71 (170) | 180 (86) | 2.6 (420) | 5.0 (580) | 10 |
| 2mg Ro48-6791 | 41 (86) | 230 (44) | 5.6 (120) | 4.8 (180) | 5 |
| 3mg Ro48-6791 | 42 (130) | 270 (81) | 6.4 (330) | 3.5 (130) | 5 |
| 1mg Ro48-8684 | 15 (110) | 630 (1000) | 43 (1700) | 0.23 (4) | 10 |
| 3mg Ro48-8684 | 9.5 (58) | 3500 (100) | 370 (130) | 0.79 (1400) | 5 |
| 10mg Ro48-8684 | 6.1 (55) | 740 (260) | 120 (330) | 0.88 (1400) | 5 |
| midazolam 0.1mg/kg | 3.0 (87) | 750 (410) | 250 (1300) | 0.75 (1200) | 20 |

**Table IV.** Percentage increase in $S_0$ (with 95% confidence interval)

| Contrast | | % increase | 95% confidence interval |
|---|---|---|---|
| from 3mg Ro48-6791 | to 1mg Ro48-6791 | 69% | (-53% - 510%) |
| from 10mg Ro48-8684 | to 1mg Ro48-8684 | 140% | (8.1% - 430%) |
| from 0.1mg/kg midazolam | to 3mg Ro48-6791 | 1300% | (320% - 4500%) |
| from 0.1mg/kg midazolam | to 10mg Ro48-8684 | 100% | (5.9% - 290%) |

The back-transformed difference equals the ratio of the geometric means and is used to provide an estimate of percentage increase with the corresponding 95% confidence interval.

*Application: results*

Parameters for the three highest doses of the new benzodiazepines and for midazolam are presented in Table III. Table IV gives the results for the comparison of the $S_0$ parameter between drugs and doses. Figure 3 provides individual concentration-effect plots chosen as an illustration of the types of concentration-effect relationships encountered.

Ro 48-6791 is much more potent than midazolam as illustrated by the 14-fold higher $S_0$ (1300% higher; 95%CI: 320% - 4500% higher). Ro 48-8684 may be somewhat more potent than midazolam with an $S_0$ that is 100% higher (95% CI: 5.9% - 290%).

There are no clear indications of a change in sensitivity for the different doses of Ro 48-6791. For Ro 48-8684 however, the $S_0$ for the 1mg group is 140% higher than in the 10mg group (95% confidence interval: 8.1% - 430%).

## Discussion

The original papers describing the PK/PD of Ro 48-6791 [3] and Ro 48-8684 [4] both encountered problems in the comparison of concentration-effect parameters.

For Ro 48-6791 *versus* midazolam, a number of subjects were best described by a linear concentration-effect model while others required an $E_{max}$ model. The resulting parameters could not be compared or pooled and drugs had to be compared on the basis of concentrations associated with the moment of reaching conscious sedation. The current approach allows the description of all relationships using the same parameters and provides a coherent set of estimates that indicate 14-fold higher potency for Ro 48-6791 than for midazolam.

For the study with Ro 48-8684, all subjects were adequately described using a linear model, even for the midazolam occasions. The comparison of slopes between the different doses of Ro 48-8684 indicated a decrease in sensitivity with increasing dose. Doubt remained however, that this difference in sensitivity was due to gradual development of $E_{max}$

behaviour for the higher doses resulting in biased slope estimates. The new method confirms the finding of a change in sensitivity.

The simulation results indicate a bias in the $S_0$ estimates in the same general direction as the change in sensitivity for Ro 48-8684, which could explain the significant decrease in sensitivity with increasing dose. This bias should however be viewed in relation to the variability of the parameter estimates as large bias is associated with high variability. This high variability makes it unlikely that a significant test outcome will be obtained when comparing increasing doses. The power for obtaining a significant test result when comparing the different doses using 5 subjects per group for the data from the simulations, is in the order of 8 to 16%. This low power makes it unlikely that the significant decrease in sensitivity to Ro 48-8684 is due to bias in the estimation method alone. Nevertheless, one should be careful in concluding that the change in sensitivity is real, as confounding factors like an anticipation to the effects of the drug over the course of the study may play a role.

In the case where low doses are compared to high doses, it is obvious that the parameters estimated for the low doses are less accurately estimated and therefore more variable than the parameters for high doses. The choice of a statistical method for comparing the groups should reflect this, and for this reason two-sample Student-s t-tests were used assuming unequal variances. Although all doses and drugs could have been put into a single ANOVA model, this would result in severe violation of the assumption of homogeneity of variances.

The use of the $S_0$-methodology is necessarily restricted to linear and $E_{max}$ concentration-effect relationships. If a drug is encountered that exhibits some sort of threshold phenomenon, that could for instance require use of a sigmoid $E_{max}$ model, other approaches should be attempted. An exponential model could be used for instance [13,14] but the parameters that result from such a model cannot be translated to the sigmoid $E_{max}$ model parameters. An alternative parameterisation of the sigmoid $E_{max}$ model that can be used for truncated data is suggested by Bachman and Gillespie [15]. This is implemented by estimating the effect (E*) corresponding to a fixed concentration (C*) that is observed on the truncated curve and by estimating a parameter $\beta$ equal to $C^*/EC_{50}$. E* and â have improved estimating properties over $E_{max}$ and $EC_{50}$ and can be useful alternatives to describing the truncated curves.

The question arises how the $S_0$ parameter behaves if the model is applied to a situation where the underlying concentration-effect model is actually linear. Because the $E_{max}$ model has one more parameter than the linear model, use of the $E_{max}$ model in this case will result in more variable estimates due to over-parameterisation. Estimation of a linear model in the simulation with no effect compartment, residual variability of 750 and a 20 min. infusion (HR0) indicates that bias clearly develops with increasing coverage of the $E_{max}$ curve but also that the slope estimates for the lowest part of the curve (with the lowest dose) are only slightly biased and about three times less variable than the corresponding $S_0$ estimates. It may therefore be tempting when analysing real-life curves, to use a linear model when the data 'look' linear (where slope substitutes $S_0$) and an $E_{max}$ model when they do not. Alternatively, a formal test like the extra sum of squares principle [16] could indicate whether the $E_{max}$ model provides a significantly better fit; the $S_0$ parameter could be used if this is the case, and the

linear slope otherwise. However, application of this principle to the data from the simulation indicates that this does not significantly decrease overall variability. This is because the occasional extreme $S_0$ estimates at the lower doses are associated with seemingly highly nonlinear concentration-effect data. Even though the data arise from a nearly linear part of the $E_{max}$ model, they are sometimes (due to measurement error) significantly better described by an $E_{max}$ model with extreme parameters, especially when only a small part of the curve is available. Substitution of $S_0$ estimates by linear slopes is therefore only feasible if external information is present indicating that an essentially linear part of the concentration-effect curve is estimated, irrespective of what the data might indicate. As this is rarely the case in practice, the $E_{max}$ model with the $S_0$ parameter should be routinely applied to all subjects and doses and not just to the ones indicating deviation from linearity.

It may be argued that the $S_0$ parameter only describes a small aspect of the concentration-effect curve and that initial sensitivity may not be a parameter of interest. However, $S_0$ is inversely related to $EC_{50}$ and may therefore be used just as well as a sensitivity parameter. Moreover, the examples indicate that situations exist where almost the entire concentration-effect curve may be described by a close-to-linear model.

The notion that $E_{max}$ and $EC_{50}$ are not the best choice for estimating the (full) $E_{max}$ model has been expressed before. Alternative parameterisations of the classic $E_{max}$ model have been advocated by Ratkowsky [17] resulting in parameters with more favourable estimating properties. These parameters are $1/E_{max}$ and $EC_{50}/E_{max}$ (which equals $1/S_0$). Examination of these 'Ratkowsky' parameters for the eight simulations in this paper does indicate less skewed distributions than for $E_{max}$ and $EC_{50}$. However, if we calculate the average absolute skewness over the five $C_{max}$-values for the eight simulations, then for seven of the simulations, the skewness is (substantially) lower for $\log(S_0)$ than for $1/S_0$. The skewness is similar for $\log(E_{max})$ and $1/E_{max}$. This indicates that use of (log) $S_0$ need not be restricted to $E_{max}$ models without a maximum, but provides a sensible alternative to $EC_{50}$ in all cases.

## Conclusions

By parameterising the $E_{max}$ model in terms of $E_{max}$ and $S_0$, close to linear concentration-effect relations may be effectively estimated, enabling comparison to situations with a more fully developed $E_{max}$ model.


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Chapter 6

# Analysis of asymmetrical agonist concentration-effect curves

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**Summary**

We have analysed the shape of concentration-effect (E/[A]) curves obtained with noradrenaline, phenylephrine, methoxamine, oxymetazoline, cirazoline, indanidine, ST 587 and SK&F 89748-A in rat aorta. A fitting procedure, based on nonlinear mixed effect modelling and original work by Richards [1], was developed to describe the degree of symmetry of E/[A] curves.

All of the agonists investigated produced concentration-dependent contractions. The four-parameter Richards model provided a significantly better fit of the E/[A] data than the standard logistic/Hill model for all ligands investigated, which implies that E/[A] curves were asymmetrical. With the exception of ST 587, the asymmetry parameter ($\delta$) tended towards zero and the Richards model could be replaced without significant loss of goodness-of-fit by the three-parameter, asymmetrical Gompertz model.

Pre-treatment with the irreversible antagonist, phenoxybenzamine (60 nM), produced a shift of the $\delta$ estimate for noradrenaline from zero to unity, indicating a change from an asymmetrical to a symmetrical curve.

This analysis demonstrates that the behaviour of $\alpha_1$-adrenoceptor agonists in rat aorta is not consistent with expectations for a simple action at a single-receptor/single transducer system. Comparison of intrinsic activities estimated in the present study with those reported for cloned $\alpha_1$-adrenoceptors suggests that the $\alpha_{1D}$- and $\alpha_{1A}$-subtypes operate in rat aorta.

The curve-fitting analysis developed in this study provides a quantitative and sensitive measure of asymmetry and a general method for the objective discrimination of agonist action on the basis of curve shape.

## Introduction

Three subtypes of $\alpha_1$-adrenoceptors, known as $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$-adrenoceptors, have been identified by molecular cloning and expression techniques. These gene products exhibit distinct pharmacological profiles as judged by radioligand binding and functional studies of tissue preparations and recombinant expression assays [2]. In addition to this molecular-based subclassification, the term $\alpha_{1L}$-adrenoceptor has been introduced to account for the low potency displayed by several competitive antagonists in various smooth-muscle preparations [3-5]. For example, prazosin has been consistently reported to exhibit affinity ($K_B$) values of more than 1 nM for the $\alpha_1$-adrenoceptors in the lower urinary tract of man [6], rat [7], dog [8] and rabbit [9], while it expresses subnanomolar affinity at the $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$ subtypes [6].

The $\alpha_1$-adrenoceptors mediating contraction of rat aorta have been investigated extensively (see Van der Graaf *et al.* [10] for a review) and their pharmacological characteristics have been subject to controversy since the first studies by Ruffolo and co-workers in the early eighties [11-13]. In recent years, several groups have suggested that the $\alpha_{1D}$-adrenoceptor is the functional $\alpha_1$-adrenoceptor in rat aorta [2,14-19]. However, on the basis of an analysis of the effects of competitive antagonists on the contractile responses to noradrenaline and phenylephrine, we have provided evidence for a heterogenous $\alpha_1$-adrenoceptor population in this tissue [10]. This hypothesis was recently confirmed and extended by Fagura *et al.* [20] and supported by Deng *et al.* [21] who reported biphasic displacement of [$^3$H]prazosin by BMY 7378 in the rat aorta. In our study, the first indication of complexity was given by small, but significant, systematic deviations of the curve fit obtained with the Hill equation from the noradrenaline and phenylephrine concentration-effect (E/[A]) curves as though the curves were not monophasic [10]. Therefore, in an attempt to illuminate further the characteristics of the functional $\alpha_1$-adrenoceptors in rat aorta, in the present study we have analysed the sigmoidal E/[A] relationships of a series of $\alpha_1$-adrenoceptor agonists. For this purpose, we have developed an objective test to determine whether the data can be best described by a symmetrical or asymmetrical model. In an attempt to maximise the chance of exposing heterogeneity, compounds were selected from different chemical classes [see 22,23]: three phenethylamines (noradrenaline, phenylephrine and methoxamine), two imidazolines (oxymetazoline and cirazoline), two imidazolidines (indanidine and ST 587) and

an aminotetralin (SK&F 89748-A).

Preliminary accounts of these data [24] and the analysis [25] have been presented to the British Pharmacological Society.

## Methods

### *Rat isolated aortic ring preparation*

Male Wistar rats (225-300 g) were killed by cervical dislocation and the thoracic aorta was dissected. The aorta was mounted on a length of scoured, polythene tubing and placed in a Petri dish containing modified Krebs-Henseleit solution (KHS) of the following (mM) composition: NaCl 119.0, $NaHCO_3$ 25.0, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, glucose 11.0, $CaCl_2$ 0.25 and ascorbic acid 0.1. The aorta was cleared from surrounding adipose tissue and the endothelium was removed by gentle rubbing of the intimal surface with the polythene tube. The effectiveness of this procedure was confirmed after completion of each agonist E/[A] curve by the lack of relaxant response to 10 ìM 5-methylfurmethide, the acetylcholine M-receptor agonist. Six ring segments (~4 mm length) were prepared from each aorta and mounted between two stainless-steel wires in 20 ml organ baths, thermostatically controlled at 37 " 0.5EC, containing modified KHS and continuously gassed with 95% $O_2$ and 5% $CO_2$. Tissue responses were measured continuously as changes in isometric tension (g) using Grass FT03C strain gauges and displayed on potentiometric chart recorders.

### *Experimental protocol*

Following application of 2 g resting tension, tissues were allowed to stabilise for 60 min during which time the organ bath fluid was replaced four times with pre-warmed KHS at regular intervals. The resting tension was re-established once after 30 min. In our rat aorta assay, the contractile effect of maximally-effective concentrations of $\alpha_1$-adrenoceptor agonists cannot be reversed readily by washing [26] and therefore only one E/[A] curve was obtained in each tissue. In order to be able to compare the intrinsic activities of different agonists with this single-curve design, tissues were calibrated with a sub-maximally effective concentration (1 μM) of phenylephrine followed by a 60 min washout period. Following 90 min incubation with 30 μM cocaine and 6 μM timolol, single agonist E/[A] curves were obtained by cumulative dosing at half-log unit concentration increments to noradrenaline, phenylephrine, methoxamine, oxymetazoline, cirazoline, indanidine, ST 587 and SK&F 89748-A.

The effects of the irreversible antagonist, phenoxybenzamine (PBZ) on the noradrenaline E/[A] relationship were also investigated. For the study of irreversible receptor antagonism, tissues were exposed to 60 nM PBZ for 4 or 7 min and washed for 30 min prior to the 90 min incubation with cocaine and timolol. PBZ did not produce any effects on basal tone.

Effects were expressed as percentage of the phenylephrine calibration response. Only one agonist E/[A] curve was obtained in each tissue.

## Analysis

*Concentration-effect models*

Initially, individual agonist E/[A] curve data were fitted to the logistic model with the following parameterisation [see 27]:

$$E = \frac{\alpha}{1 + e^{-\ln(10)\_p\_(\log[A] - \log(EC_i))}} \qquad (1)$$

to provide estimates of the upper asymptote (α), the point of inflection ($pEC_i$, that is $-\log_{10}(EC_i)$) and the slope parameter (p) at the point of inflection. The point of inflection is at 50% of á (i.e. $pEC_i = pEC_{50}$). This form of the logistic model is equivalent to the Hill equation [28] and p is equivalent to the Hill slope parameter, $n_H$.

Subsequently, in an attempt to quantify the apparent asymmetry of the E/[A] curves, the data were fitted to the four-parameter Richards model which includes an asymmetry factor, δ [1]:

$$E = \frac{\alpha}{(1 + \delta\_ e^{-\ln(10)\_p\_(\log[A] - \log(EC_i))})^{1/\delta}} \qquad (2)$$

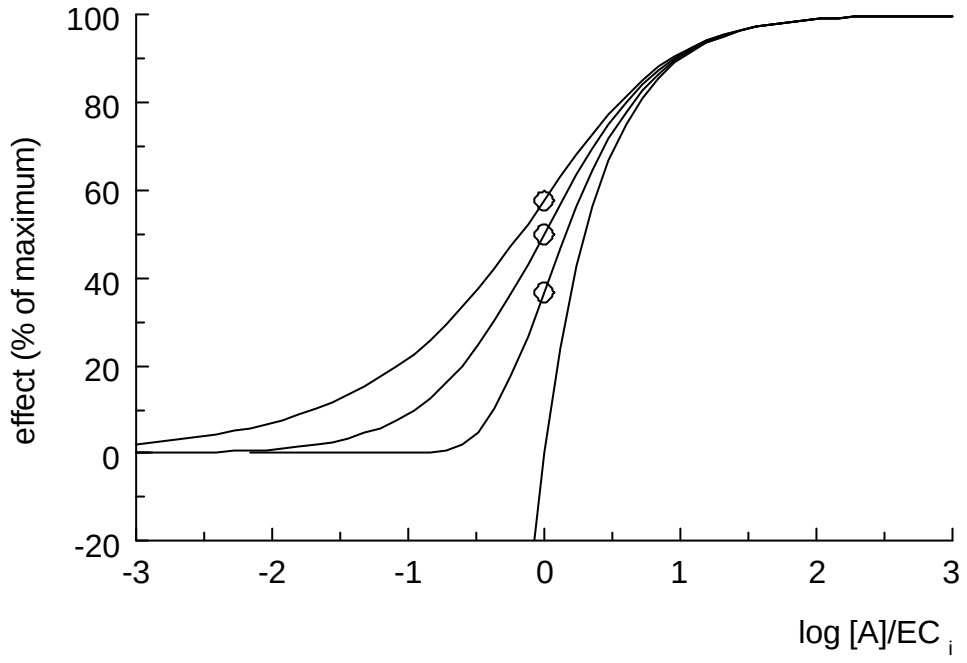


**Figure 1.** Richards curves with from left to right δ=2,1,0 and -1; p = 1, α=100 and $pEC_i$= 0; open circles are the points of inflection.

When δ=1, this equation is identical to the logistic model (Equation 1) and describes a symmetrical E/[A] curve; in all other cases the Richards model is asymmetrical (Figure 1). When δ approaches 0, the Richards model collapses to the three-parameter Gompertz model

$$\lim_{\delta \to 0} (1 + \delta \bullet x)^{1/\delta} = e^{x} \qquad (3)$$

[see 1,27], which can be derived using the standard limit:
This means that for δ approaching 0:

$$E = \frac{\alpha}{e^{e^{-\ln(10) \bullet p \bullet (\log[A] - \log EC_i)}}} \qquad (4)$$

Like the Richards model, the Gompertz model is asymmetrical about its point of inflection, which occurs at $\alpha/e$ (~37% of the maximum effect; Figure 1). The midpoint location ($pEC_{50}$) of the Gompertz model can be calculated as follows:

$$pEC_{50} = pEC_i - \frac{\ln(\ln(2))}{p} \qquad (5)$$

When $\delta$=-1, the model collapses to the "monomolecular function" [1], which has no lower asymptote and point of inflection and thus allows infinite and negative values of E for low agonist concentrations (Figure 1):

$$E = a \bullet (1 - e^{-\ln(10) \bullet p \bullet (\log[A] - \log EC_i)}) \qquad (6)$$

When -1<$\delta$<0, the Richards model describes E/[A] curves that are not monotonic with infinite high values of E for low agonist concentrations. Therefore, we regarded the E/[A] profiles described by the Richards model with negative values of $\delta$ as lacking pharmacological meaning for the present study and only the condition $\delta$ approaching 0 was considered in the analysis of the experimental data.

*Nonlinear mixed effect modelling*

With conventional nonlinear least-squares regression, individual parameters are estimated for each E/[A] curve, so that a data set of n individual E/[A] curves would result in 3n or 4n parameter estimates for a three- and four-parameter model, respectively. Due to high intrinsic nonlinearity, the Richards model has very poor statistical properties in estimation and individual parameter estimates are difficult to obtain [27]. Therefore, conventional nonlinear least-squares regression was not suitable for the present analysis and we employed nonlinear mixed effect modelling (see [29], for details). With this method, no individual parameters are estimated for each E/[A] curve. Instead, the model parameters for each individual E/[A] curve are assumed to originate from a common distribution and only the mean and inter-individual variability are estimated. This means that, irrespective of the number of individual E/[A] curves in a data set, only 6 and 8 estimates (i.e. the mean parameters and associated inter-individual variabilities) are obtained for a three- and four-parameter model, respectively. The advantage of nonlinear mixed effect modelling is that all data are fitted simultaneously in such a manner that combining
of information of each individual experiment may allow adequate estimation of ill-defined parameters.

Although nonlinear mixed effect modelling itself does not provide individual parameter estimates, so-called empirical Bayes estimates can be generated for each separate E/[A] curve (see [29] for details). These estimates are Bayesian in the sense that they are

conditional on the population information and empirical because the population information is not external to the data, but based on the data itself.

*Model discrimination*

In order to determine whether two models yield significantly different goodness-of-fits for the same data set, standard test theory [30,31] requires that they are nested so that one model can be formulated as a special case of the other one by setting one or more of the parameters to fixed values. As shown above, the three-parameter logistic and Gompertz models are sub-models of the four-parameter Richards model when $\delta$ approaching 1 and 0, respectively. Thus, a fit with the Richards model can be compared to each of the three-parameter models using standard test theory. When conventional nonlinear least-squares regression would have been used, the significance of the increase in goodness-of-fit with the Richards model could have been tested using the extra-sum-of-squares principle [30], where the variance reduction due to the inclusion of extra parameters is compared with the residual variance of the full model using an F-distribution. In the present study, however, maximum-likelihood estimation was used for the nonlinear mixed effect modelling and nested models were compared using the minimum value of the objective function (MVOF), which is equal to -2*log likelihood. Using likelihood ratio theory [31] it can be shown that the difference between the MVOFs for two nested models follows a chi-square distribution with degrees of freedom equal to the difference in the number of parameters. In the present study, the asymmetry parameter ($\delta$) was estimated without inter-individual variability and therefore the comparison between the Richards and the three-parameter models is based on a single-degree of freedom, which is significant at the $\alpha$=5% level when the MVOF difference exceeds 3.85.

Since the logistic and Gompertz models are not nested, it is not possible to test directly whether one of the models provides a significantly better fit than the other. However, the improvement of the fit with the Richards model can be tested relative to both models and this can be used as an indirect test to compare the logistic and Gompertz models. In the most extreme case, one of the three-parameter models is indistinguishable from the Richards models (i.e. the estimate of $\delta$ is not significantly different from zero or unity) while the other three-parameter model is associated with a significant decrease in the goodness-of-fit. This outcome can be interpreted as a significant better goodness-of-fit for one of the three-parameter models compared to the other. However, when the Richards model provides a significantly better fit than both three-parameter models, no formal statement can be made with regard to the difference between the performance of the logistic and Gompertz model.

*Software*

All fitting procedures were performed by use of the nonlinear mixed effect modelling software

package NONMEM (GloboMax LLC, Hanover, Md, USA). An IBM-compatible personal computer (Pentium$^7$ 133 MHz) running under Windows 3.11 was used with the Microsoft FORTRAN PowerStation 1.0 compiler and NONMEM version IV, level 2.0 (double precision). Parameters and associated standard errors (s.e.) were estimated using first-order conditional estimation and additive intra-individual and multiplicative inter-individual residual error models were assumed [29]. Estimates of the inter-individual variability were expressed as coefficient of variation (CV). For computational reasons, a lower boundary value between $10^{-2}$ and $10^{-5}$ was imposed on $\delta$. The NONMEM syntax is provided in the Appendix.

*Compounds*

Compounds were obtained from the following sources: methoxamine hydrochloride, (-)-noradrenaline hydrochloride, (-)-phenylephrine hydrochloride, oxymetazoline hydrochloride, phenoxybenzamine hydrochloride and cocaine hydrochloride (Sigma Chemical Company Ltd., U.K.); ST 587 (2-(2-chloro-5-trifluoromethylphenylimino)-imidazolidine nitrate; a gift from Boehringer Ingelheim, Germany); timolol maleate (Merck, Sharp & Dohme, U.K.); indanidine hydrochloride (also known as Sgd 101/75; a gift from Dr. U. Jahn, Siegfried Ltd., Switzerland); SK&F-l-89748-A (l-1,2,3,4-tetrahydro-8-methoxy-5-(methylthio)-2-naphthalenamine hydrochloride; a gift from SmithKline Beecham Pharmaceuticals, U.S.A.); cirazoline hydrochloride (a gift from Synthélabo Recherche (L.E.R.S.), France); 5-methylfurmethide iodide (James Black Foundation, U.K.).

Noradrenaline and phenylephrine were dissolved and diluted in stoichiometric aqueous ascorbic acid solution to prevent oxidation. Phenoxybenzamine was dissolved in absolute ethanol. SK&F 89748-A was dissolved initially in 50% ethanol to give a 0.2 mM stock solution and was subsequently diluted in distilled water. All other drugs were dissolved in distilled water. Noradrenaline and phenylephrine were made up freshly each day. All other drug stock solutions were stored below –20 C and diluted on the day of the experiment. The maximum volume of drug solution administered to the 20 ml organ baths did not exceed 800 µl, corresponding to 4% of the bath volume.

## Results

*$\alpha_1$-Adrenoceptor concentration-effect relationships*

All of the agonists investigated produced concentration-dependent contractions of the aorta (Figure 2). Initially the individual E/[A] curve data for each ligand were fitted to the logistic model (Equation 1) to obtain estimates of $pEC_{50}$, p (which is equivalent to the Hill slope parameter) and $\alpha$ (Table I). Although convergence was obtained for each agonist, in most cases the model predictions appeared to deviate systematically from the individual E/[A]

data, consistent with our observation in the previous study [10]. The deviations were difficult to detect by eye but most notable at concentrations producing ~60-80% of the maximum response ($\alpha$), as illustrated by an individual noradrenaline E/[A] curve in Figure 3b. In an attempt to quantify the apparent asymmetry of the E/[A] curves in an objective manner, we re-fitted the E/[A] data to the four-parameter Richards model (Equation 2). As judged by the decrease in the minimum value of the objective function, the fit obtained with the Richards model was significantly better than that to the logistic model for all the agonists tested (Table I). With the exception of ST 587, the estimates of the asymmetry parameter $\delta$, reached the lower boundary value or were not significantly different from 0 (oxymetazoline, Table I). Therefore, the data were subsequently also fitted to the three-parameter Gompertz model (Equation 4), which is identical in algebraic formulation to the Richards model when $\delta$ approaching 0 (see Methods). Table I shows that the minimum value of the objective function obtained with the Gompertz and Richards models were practically identical for all agonists except ST 587. In the case of ST 587, the goodness-of-fit for the Gompertz model was significantly lower than for the Richards model. As an example, the fits obtained for noradrenaline with the logistic and Gompertz model are shown in Figure 3 for an individual E/[A] curve and for the average data.

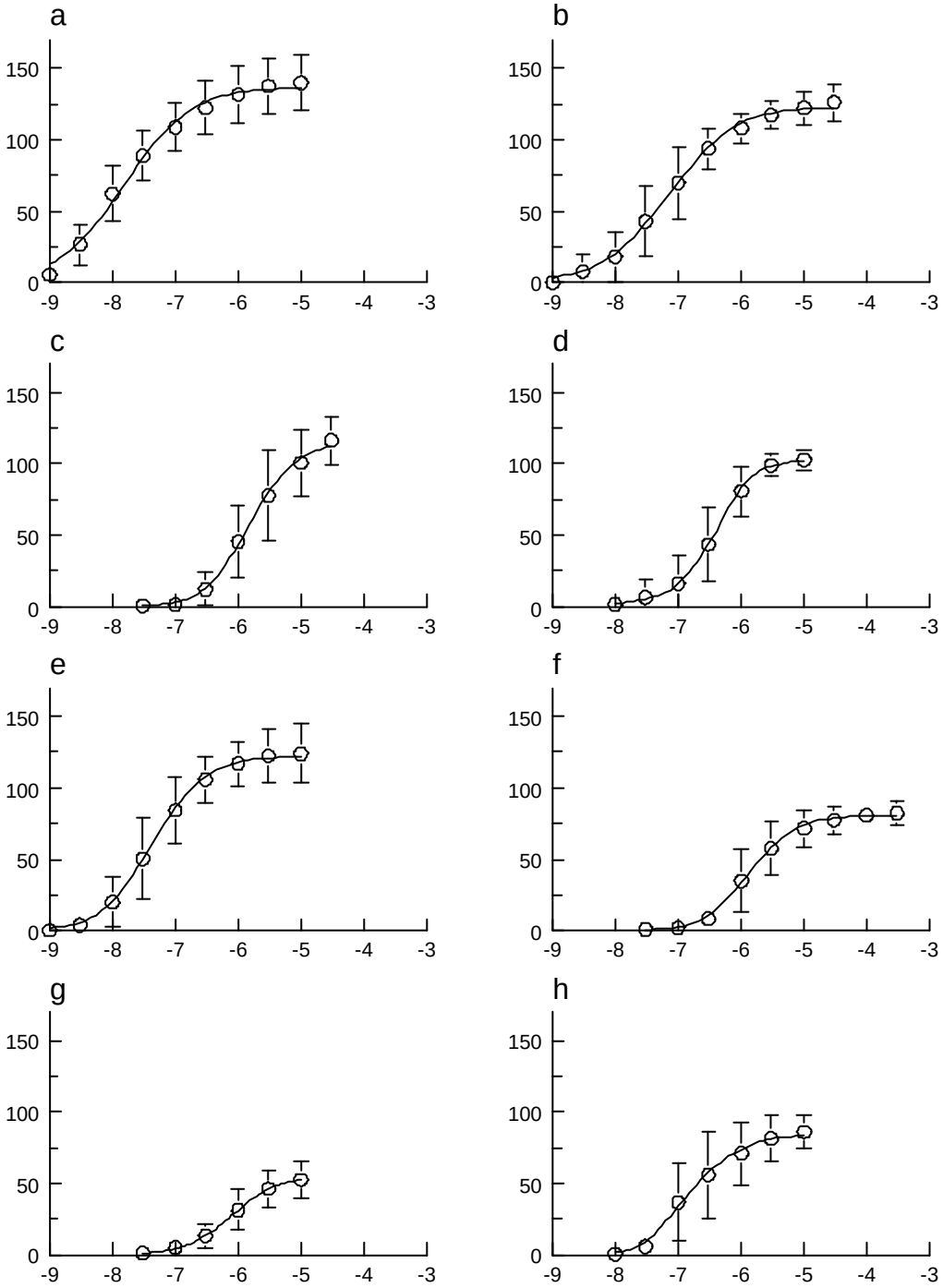


**Figure 2.** Concentration-effect curves obtained on rat aorta to (a) noradrenaline, (b) phenylephrine, (c) methoxamine, (d) oxymetazoline, (e) cirazoline, (f) indanidine, (g) ST587 and (h) SK&F 89748-A. The curves shown superimposed on the mean experimental data points were obtained by averaging the individual fits with the logistic-model (Equation (1)). Error bars indicate standard deviation. Abscissae: [agonist] (log M). Ordinates: effect (% of 1 μM phenylephrine calibration response).

**Table I.** Parameter estimates (mean ± s.e.) obtained by simultaneously fitting individual agonist concentration-effect curves to the logistic, Richards and Gompertz model using nonlinear mixed-effect modelling. Estimates of inter-individual variability are shown as CV in parentheses.

| Agonist[1] | Model | $pEC_i$ | p | $\alpha$[2] | $\delta$ | MVOF[3] |
|---|---|---|---|---|---|---|
| Noradrenaline | Logistic | 7.81 ± 0.08 (3%) | 0.86 ± 0.03 (0%) | 136 ± 6 (13%) | - | 465.7* |
| (n=10) | Richards | 8.10 ± 0.08 (3%) | 0.57 ± 0.03 (13%) | 140 ± 6 (13%) | 0[#] | 386.2 |
| | Gompertz | 8.11 ± 0.08 (3%) | 0.57 ± 0.03 (13%) | 140 ± 6 (13%) | - | 386.2 |
| Phenylephrine | Logistic | 7.17 ± 0.15 (6%) | 0.97 ± 0.08 (22%) | 122 ± 4 (9%) | - | 404.2* |
| (n=8) | Richards | 7.44 ± 0.16 (6%) | 0.63 ± 0.06 (24%) | 127 ± 5 (10%) | 0[#] | 364.4 |
| | Gompertz | 7.44 ± 0.16 (6%) | 0.63 ± 0.06 (24%) | 127 ± 5 (10%) | - | 364.4 |
| Methoxamine | Logistic | 5.80 ± 0.13 (6%) | 1.51 ± 0.14 (19%) | 114 ± 6 (14%) | - | 257.3* |
| (n=7) | Richards | 5.96 ± 0.14 (6%) | 0.95 ± 0.10 (24%) | 120 ± 7 (15%) | 0[#] | 237.5 |
| | Gompertz | 5.96 ± 0.14 (6%) | 0.94 ± 0.10 (25%) | 121 ± 7 (15%) | - | 237.1 |
| Oxymetazoline | Logistic | 6.47 ± 0.13 (5%) | 1.76 ± 0.26 (37%) | 102 ± 3 (7%) | - | 238.3* |
| (n=7) | Richards | 6.61 ± 0.20 (6%) | 1.21 ± 0.25 (37%) | 105 ± 2 (7%) | 0.14 ± 0.32 | 229.9 |
| | Gompertz | 6.64 ± 0.15 (6%) | 1.13 ± 0.16 (37%) | 105 ± 5 (7%) | - | 230.3 |
| Cirazoline | Logistic | 7.39 ± 0.13 (5%) | 1.33 ± 0.08 (15%) | 120 ± 6 (15%) | - | 395.8* |
| (n=9) | Richards | 7.58 ± 0.13 (5%) | 0.89 ± 0.06 (18%) | 123 ± 7 (16%) | 0[#] | 349.0 |
| | Gompertz | 7.58 ± 0.13 (5%) | 0.89 ± 0.06 (18%) | 123 ± 7 (16%) | - | 349.0 |
| Indanidine | Logistic | 5.84 ± 0.12 (5%) | 1.42 ± 0.06 (0%) | 80 ± 3 (8%) | - | 206.5* |
| (n=6) | Richards | 6.02 ± 0.12 (5%) | 0.96 ± 0.05 (7%) | 82 ± 3 (8%) | 0[#] | 183.2 |
| | Gompertz | 6.02 ± 0.12 (5%) | 0.96 ± 0.05 (7%) | 82 ± 3 (8%) | - | 183.2 |
| ST 587 | Logistic | 6.11 ± 0.10 (4%) | 1.34 ± 0.12 (20%) | 54 ± 6 (25%) | - | 126.7* |
| (n=6) | Richards | 6.04 ± 0.10 (4%) | 1.76 ± 0.41 (19%) | 52 ± 6 (25%) | 1.70 " 0.46 | 122.1 |
| | Gompertz | 6.26 ± 0.12 (4%) | 0.73 ± 0.07 (13%) | 59 ± 6 (24%) | - | 145.6* |
| SK&F 89748-A | Logistic | 6.72 ± 0.19 (6%) | 1.57 0.12 (10%) | 84 ± 3 (11%) | - | 199.9* |
| (n=6) | Richards | 6.88 ± 0.19 (7%) | 0.96 0.10 (23%) | 88 ± 3 (8%) | 0[#] | 176.1 |
| | Gompertz | 6.88 ± 0.19 (7%) | 0.96 0.10 (23%) | 88 ± 3 (8%) | - | 176.1 |

[1]Number of replicates shown in parentheses.

[2]Expressed as percentage of a 1 μM phenylephrine calibration response

[3]Minimum value of the objective function

[#]Lower boundary value ($10^{-5}$-$10^{-2}$)

*Goodness-of-fit significantly lower than goodness-of-fit to the Richards model (P<0.05).

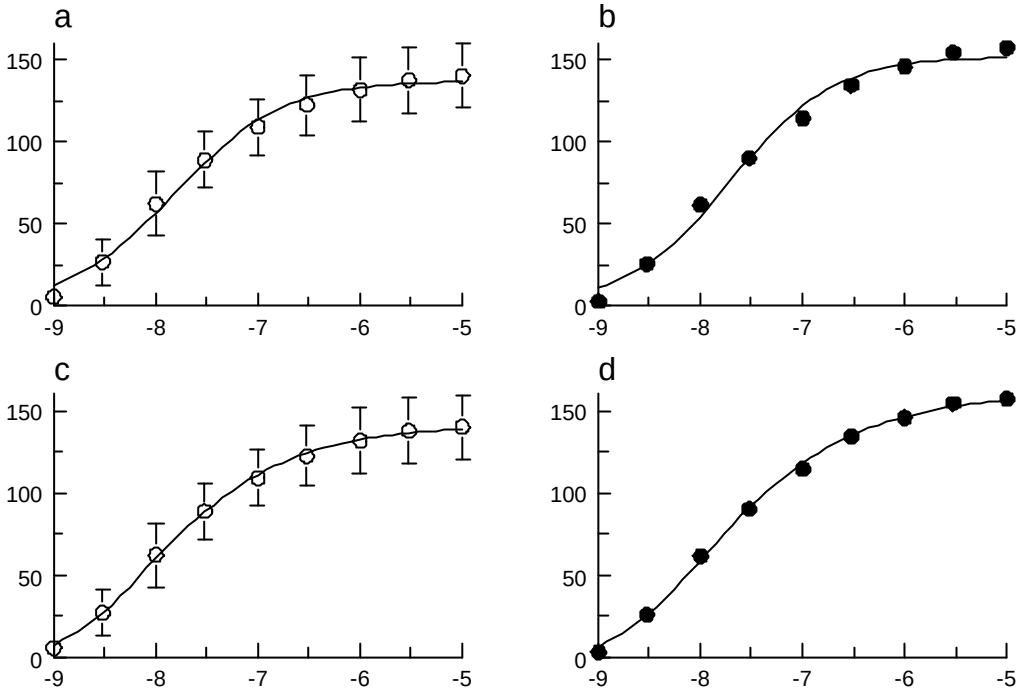


**Figure 3.** Comparison of the fits obtained with the logistic (a and b) and Gompertz (c and d) model for the mean noradrenaline concentration-effect data (a and c) and for a representative, individual noradrenaline curve (b and d) obtained on rat aorta. The curves shown superimposed on the mean (n = 10) experimental data points were obtained by averaging the individual fits with the logistic (a) and Gompertz (c) model. Error bars indicate standard deviation. The curves shown superimposed on the data points from the individual experiment were simulated with the following empirical Bayes parameter estimates for the logistic (b) and Gompertz (d) model: $pEC_i$ = 7.56 and 7.84; p = 0.86 and 0.60; $\alpha$= 150 and 154, respectively. Abscissae: [noradrenaline] (log M). Ordinates: effect (% of 1 μM phenylephrine calibration response).

Subsequently, we investigated the effects of reducing efficacy on the E/[A] curve shape. For this purpose, an experiment was performed to study the interaction between noradrenaline and the irreversible antagonist PBZ.

*Analysis of the interaction between phenoxybenzamine and noradrenaline*

Pre-treatment with the irreversible $\alpha$-adrenoceptor antagonist, PBZ (60 nM for 4 and 7 min followed by 30 min washout), produced a rightward shift and depression of the

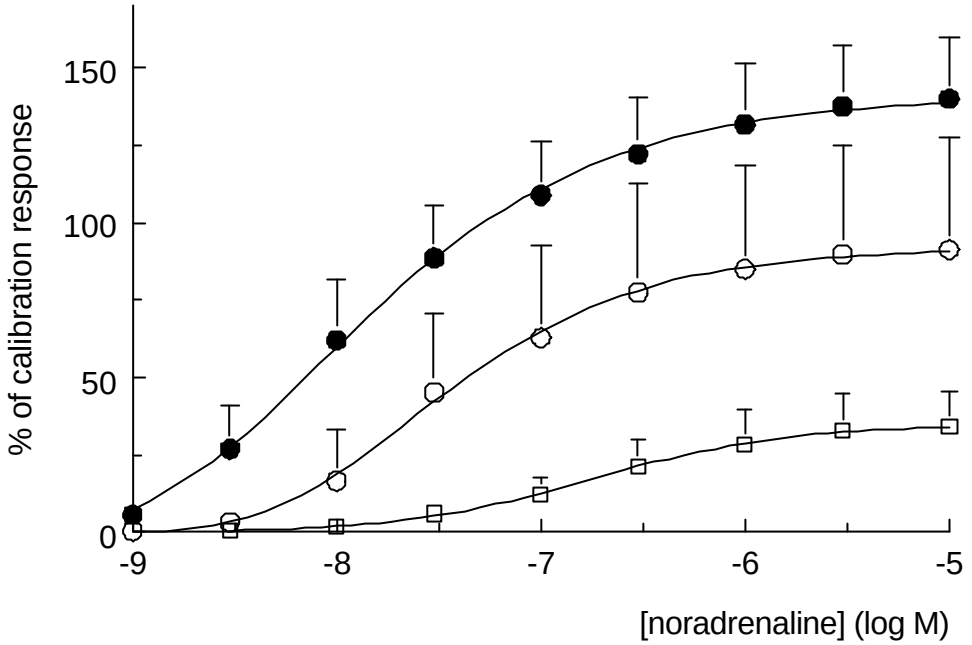


**Figure 4.** Concentration-effect curves to noradrenaline obtained on rat aorta following pretreatment with 60 nM phenoxybenzamine for 0 (**M**), 4 (**F**) and 7 (**O**) min. The curves shown superimposed on the mean experimental data points were obtained by averaging the individual fits with the Gompertz model for the 0 and 4 min pretreatment groups and with the logistic model for the 7 min pretreatment group. Error bars indicate standard deviation. Effect is expressed as percentage of a 1 μM phenylephrine calibration response.

noradrenaline E/[A] curve (Figure 4). As found previously for the noradrenaline control curves, in the case of 4 min pretreatment the data fits with the Richards and Gompertz models were identical (MVOF = 192.6; $\delta$ approaching 0, $pEC_i = 7.60 \pm 0.13$, $p = 0.70 \pm 0.02$, $\alpha = 82 \pm 20\%$ of the phenylephrine calibration response; n = 5) and significantly better than the logistic-model fit (MVOF = 221.7; $P<0.0001$). However, following 7 min PBZ pretreatment, the Richards and logistic models fitted the data equally well (MVOF = 89.1 and 89.5, respectively) while the goodness-of-fit for the Gompertz model was significantly lower (MVOF = 95.7; $P<0.05$). The asymmetry factor estimated with the Richards model was not significantly different from unity ($\delta = 0.78 \pm 0.54$) and the parameter estimates for the logistic model with $\delta$ constrained to unity were as follows: $pEC_i = 6.74 \pm 0.03$, $p = 0.94 \pm 0.04$, $\alpha = 33 \pm 5\%$ of the phenylephrine calibration response; n = 5).

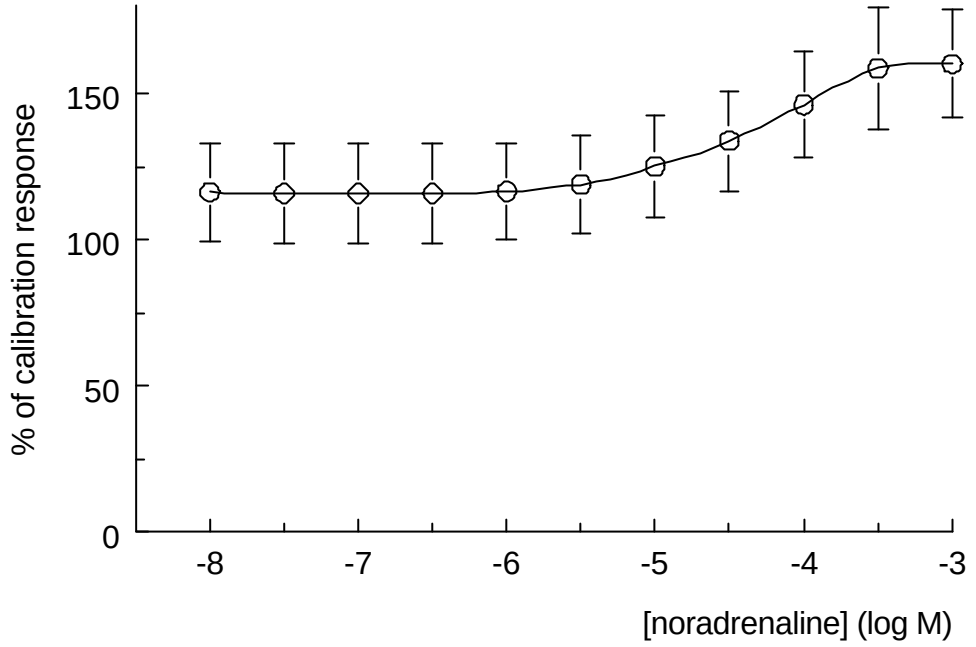


**Figure 5.** Concentration-effect curve (n=4) to noradrenaline obtained on rat aorta in the presence of 1 μM prazosin following precontraction with 75 mM KCL. Error bars indicate standard deviation. Effect is expressed as percentage of a 1 μM phenylephrine calibration response.

*Involvement of relaxant responses*

Previously, we have developed a model of functional antagonism that predicts asymmetrical E/[A] relationships and antagonist-induced changes in curve shape when an agonist acts simultaneously at two receptors mediating opposing stimuli [32]. Therefore, an attempt was made to expose a relaxant action of noradrenaline which could explain the complexity encountered in the present study. In the presence of a high concentration (1 μM) of prazosin, tissues were precontracted with 75 mM KCl, which produced a sustained response of 117.1 ± 8.2% (n = 4) compared to the phenylephrine calibration. 10 nM **B** 3 μM Noradrenaline had no significant effect on the KCl response while higher concentrations produced a further contraction (Figure 5).

## Discussion

In a previous study, we proposed that the $\alpha_1$-adrenoceptor population in rat aorta is not homogeneous [10]. This conclusion was reached by analysis, using the Hill equation, of the effects of competitive antagonists on the steepness of noradrenaline and phenylephrine E/[A] curves. Although the corresponding Schild analysis also revealed some complexities, it was shown that changes in the Hill slope of the agonist E/[A] curves were more sensitive indicators of receptor heterogeneity than deviations from linear, unit-slope Schild plots. On the basis of this finding, we wondered whether comparison of E/[A] curve shapes of different agonists could provide information about the character of the $\alpha_1$-adrenoceptors in rat aorta. In the present study, we have explored this idea and developed an objective and sensitive fitting procedure, based on original work by Richards [1] to describe and provide a discriminatory test for differences in agonist E/[A] curve shape.

The main finding of the present study can be summarised as follows. First, the Richards model provided a significantly better fit of the E/[A] data than the logistic model for all ligands investigated (Table 1), which implies that $\alpha_1$-adrenoceptor agonists from various chemical classes produce asymmetrical E/[A] curves in rat aorta. With the exception of ST 587, the asymmetry parameter ($\delta$) tended towards zero and the Richards model could be replaced without significant loss of goodness-of-fit by the asymmetrical Gompertz model, which contains the same number of parameters as the logistic model. Second, it was shown that 7 min pretreatment with 60 nM PBZ produced a shift of the $\delta$ estimate for noradrenaline from zero to unity, that is a change from an asymmetrical to a symmetrical E/[A] curve (Figure 4).

First we considered whether the asymmetrical curve shape could be due to factors other than agonist-dependent, differential activation of multiple $\alpha_1$-adrenoceptors. Differential agonist uptake and activation of other receptor classes could produce complex agonist curves and account for differences between agonists (see, for example, Van der Graaf *et al* [32]). However, cocaine and timolol were present in all experiments to block $Uptake_1$ and $\beta$-adrenoceptors, respectively, and $Uptake_2$ does not appear to play a significant role in the rat aorta assay [10]. Furthermore, the endothelium was always removed and we failed to expose a vasodilator effect of noradrenaline in the presence of $\alpha_1$-adrenoceptor blockade (Figure 5). We have also considered the possibility that the calibration of tissues with 1 $\mu$M phenylephrine influenced the E/[A] curve shape. However, when the phenylephrine calibration was omitted, noradrenaline and phenylephrine E/[A] curves were still best described by the Gompertz model with parameter estimates that were indistinguishable from the ones obtained from the experiments in which tissues were exposed to phenylephrine (data not shown).

Although it has been our experience that single-receptor systems often produce E/[A] curves that appear to be indistinguishable from logistic functions, the observed asymmetry by itself does not necessarily imply heterogeneity of $\alpha_1$-adrenoceptors in rat aorta. First, Black *et al.* [33] have shown that non-logistic E/[A] curves are predicted even when receptor occupancy is described by a rectangular hyperbolic function and the relation between occupancy and effect is assumed to be logistic. However, under these conditions the degree of asymmetry is predicted to be inversely related to agonist efficacy and deviations from the logistic model will only be experimentally detectable in the case of low-efficacy ligands [34]. However, in this study, the reduction of agonist efficacy by irreversible $\alpha_1$-adrenoceptor blockade with PBZ [35-38] produced the opposite effect. Thus, the noradrenaline E/[A] curves were asymmetrical in control tissues but became symmetrical after PBZ treatment.

Another explanation for the data would be heterogeneity of post-receptor events. For example, Ruffolo and coworkers [39-43] have suggested that the rat aorta $\alpha_1$-adrenoceptor is coupled to two signal transduction pathways; one coupled to the influx of extracellular $Ca^{2+}$ and the other to intracellular release of $Ca^{2+}$. Furthermore, it has been proposed that $\alpha_1$-adrenoceptors in rat aorta are coupled to more than one type of G-protein [44,45]. However, although mathematical models of promiscuous coupling of a single receptor to multiple transduction processes predict multi phasic E/[A] curves [39,46], they cannot account for the steepening of the noradrenaline and phenylephrine E/[A] curves by competitive $\alpha_1$-adrenoceptor antagonists observed in our previous study [10]. We have shown that the antagonist-induced curve steepening can be accounted for by a two-receptor/one-inducer model [10], but the possibility that multiple transducers also play a role cannot be excluded.

Overall, therefore, our analysis of E/[A] curve shape demonstrates that the behaviour of $\alpha_1$-adrenoceptor agonists in rat aorta is not consistent with expectations for a simple action at a single-receptor/single-transducer system. The agonists used in this study are known to display a significant degree of variation in their selectivity for $\alpha_1$-adrenoceptor subtypes [22] and it was possible that this would be mirrored in differences between E/[A] curve shapes. Although all agonists produced asymmetrical E/[A] curves, comparison of the fits suggests that they do not interact in a similar manner with the $\alpha_1$-adrenoceptors in aorta. First, for seven of the eight agonist E/[A] profiles the asymmetry parameter estimate ($\delta$) tended to zero and the Richards model could be replaced by the less complex Gompertz model in these cases without loss of goodness-of-fit (Table I). However, in the case of ST 587 the estimate of $\delta$ (1.70) was significantly greater than zero and the Gompertz model did not provide an adequate alternative for the Richards model (Table I). Thus, the analysis distinguishes the behaviour of ST 587 from that of the other ligands which suggests a different receptor-subtype selectivity. It is not clear whether this is related to the fact that ST 587 also displayed the lowest intrinsic activity (Table I). It should be noted that although the $\delta$ estimate was not significantly different from unity, the goodness-of-fit obtained with the logistic model was significantly lower than that obtained with the Richards model (Table I). A second indication of differences in agonist action is given by the finding that the slope parameter estimates (p) for the two catecholamines, noradrenaline and phenylephrine, were markedly lower than those for

the other agonists (Table I). Since phenylephrine displayed practically the same intrinsic activity as methoxamine and cirazoline (Table I), this ligand-dependency of E/[A] curve steepness cannot be simply explained by a difference in the overall efficiency of signal transduction.

An unexpected finding of this study was that asymmetrical noradrenaline E/[A] curves became symmetrical after prolonged PBZ treatment. An explanation for this observation would be that a sub-population of $\alpha_1$-adrenoceptors in aorta display greater sensitivity to alkylation by PBZ than others and that an essentially homogeneous population of PBZ-resistant receptors mediates contraction to noradrenaline after the pretreatment. As far as we are aware, PBZ has not yet been demonstrated clearly to display selectivity between the known $\alpha_1$-adrenoceptor subtypes and it is therefore difficult to interpret this result in terms of the currently accepted classification of $\alpha_1$-adrenoceptors [2]. It is generally agreed that $\alpha_{1D}$-adrenoceptors play an important role in mediating contraction to noradrenaline and other $\alpha_1$-adrenoceptor agonists in rat aorta [2,10,16-20,47]. To determine which other receptors might be involved, we compared agonist intrinsic activities estimated in the present study for contractile responses in rat aorta (Table I) with those published by Minneman *et al.* [22] for [$^3$H]inositol phosphate formation by cloned $\alpha_1$-adrenoceptor subtypes. Figure 6 shows that oxymetazoline, indanidine and ST 587 were reported to be devoid of efficacy in the rat cloned $\alpha_{1D}$-adrenoceptor assay but produced significant contraction of rat aorta. These differences in agonist activity cannot be explained simply by between-assay differences in receptor concentration and/or efficiency of coupling, because SK&F 89748-A produced ~80% of the noradrenaline maximum response at the cloned $\alpha_{1D}$-adrenoceptor but displayed practically the same intrinsic activity as oxymetazoline and indanidine in aorta (Figure 6). Thus, if it is assumed that the $\alpha_{1D}$-adrenoceptor is present aorta, the involvement of at least one other receptor is required to explain the responses to oxymetazoline, indanidine and ST 587. Since oxymetazoline, indanidine and ST 587 have been shown to express efficacy only at the cloned $\alpha_{1A}$- and not at the $\alpha_{1B}$- and $\alpha_{1D}$-subtypes [22,48], it appears that $\alpha_{1D}$- and $\alpha_{1A}$-adrenoceptors operate in rat aorta. This conclusion is in agreement with that reached by Fagura *et al.* [20]. However, it is not possible to rule out the involvement of other $\alpha_1$-adrenoceptor subtypes. For example, very recently Muramatsu *et al.* [47] have suggested that oxymetazoline mediates contraction of rat aorta through the $\alpha_{1B}$-subtype. Moreover, several studies have detected mRNA for all three cloned $\alpha_{1A}$-adrenoceptors in rat aorta [49-52].

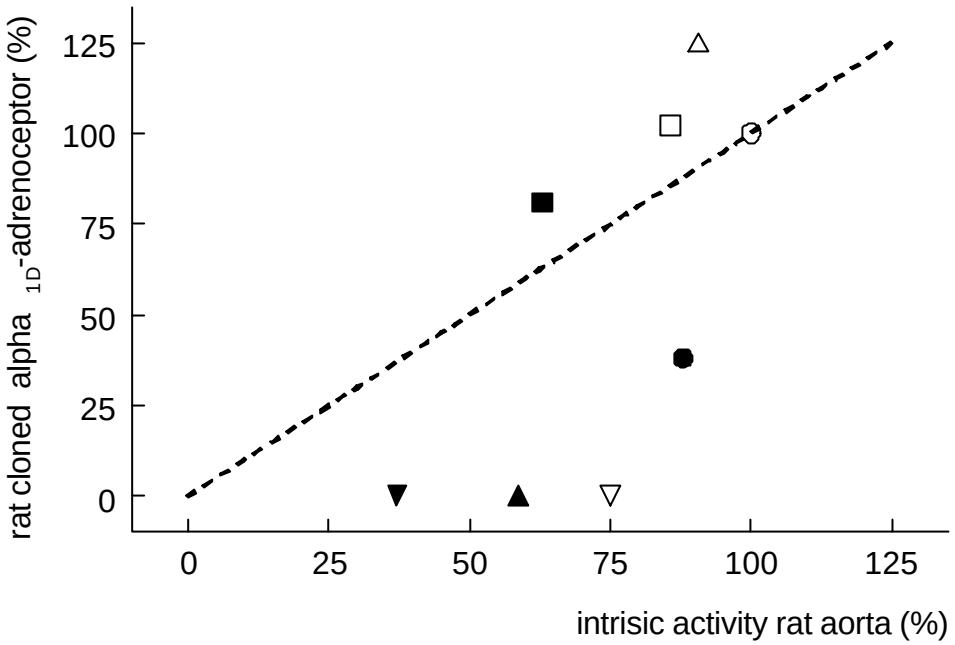


**Figure 6.** Relation between the intrinsic activity of noradrenaline (**F**), phenylephrine ( ), methoxamine (**G**), oxymetazoline (∇), cirazoline (**M**), indaninine (**–**), ST 587 ( • ) and SK&F 89748-A (**O**) for contractile responses in rat aorta (present study) and for [$^3$H]inositol phosphate formation by the rat cloned $\alpha_{1D}$-adrenoceptor expressed in human embryonic kidney 293 cells [22]. Intrinsic activity is expressed as percentage of the intrinsic activity of noradrenaline. The dashed line represents the line of identity.

Traditionally, analysis of E/[A] curves has focused on estimation of the location parameter and upper asymptote to describe agonist potency and intrinsic activity, respectively. More recently, it has been demonstrated that estimates of midpoint slope parameters obtained by fitting E/[A] curves to the Hill/logistic equation can also provide critical information for quantitative interpretation of drug-receptor interactions [10,33,53-55]. In this study, we have found that quantitative descriptors of the degree of symmetry of E/[A] curves can also contribute to pharmacological analysis. Thus, the analysis developed provides a quantitative and sensitive measure of asymmetry that cannot be obtained with the standard Hill/logistic equation and should be generally applicable for the objective discrimination of agonist action on the basis of their E/[A] shape in other assay systems.

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## Appendix

The following NMTRAN-syntax was used for the estimation of the Richards model parameters:

```
$PROB RICHARDS MODEL: CIRAZ: method=1 R_CIRAZ
$INPUT A E=DV ID
$DATA CIRAZ.DAT
$PRED
 LA = LOG10(A)
 PEC50 = THETA(1)*EXP(ETA(1))
 P = THETA(2)*EXP(ETA(2))
 ALPHA = THETA(3)*EXP(ETA(3))
 ASY = THETA(4)
 L = LA+PEC50
 LL = L*P
 DEN1 = EXP(-2.3026*LL)
 DEN2 = 1 + (ASY*DEN1)
 DEN3 = DEN2**(1/ASY)
 IF (DEN3.EQ.0) EXIT 1 20
 F = ALPHA/DEN3
 IPRE = F
 Y = F+EPS(1)
$THETA  7.4 0.8  120 (0.00001,0.5,4)
$OMEGA  1 .1 .1
$SIGMA 15
$EST MAXEVAL = 2000 PRINT=4 METHOD=1 POSTHOC NOABORT
$COV
$TABLE ID LA E PEC50 P ALPHA ASY IPRE FILE=R_CIRAZ.DAT NOHEADER NOPRINT
```

The following NMTRAN-syntax was used for the estimation of the logistic model parameters:

```
$PROB LOGISTIC MODEL: CIRAZ: method=1 L_CIRAZ
$INPUT A E=DV ID
$DATA CIRAZ.DAT
$PRED
 LA = LOG10(A)
 PEC50 = THETA(1)*EXP(ETA(1))
 P = THETA(2)*EXP(ETA(2))
 ALPHA = THETA(3)*EXP(ETA(3))
 L = LA+PEC50
 LL = L*P
 DEN1 = EXP(-2.3026*LL)
 DEN2 = 1 + DEN1
 IF (DEN2.EQ.0) EXIT 1 20
 F = ALPHA/DEN2
 IPRE = F
 Y = F+EPS(1)
$THETA  7 0.8  120
$OMEGA  1 .1 .1
$SIGMA 5
$EST MAXEVAL = 2000 PRINT=4 METHOD=1 POSTHOC NOABORT
$COV
$TABLE ID LA E PEC50 P ALPHA IPRE FILE=L_CIRAZ.DAT NOHEADER NOPRINT
```

The following NMTRAN-syntax was used for the estimation of the Gompertz model parameters:

```
$PROB GOMPERTZ MODEL: CIRAZ: method=1 G_CIRAZ
$INPUT A E=DV ID
$DATA CIRAZ.DAT
$PRED
 LA = LOG10(A)
 PEC50 = THETA(1)*EXP(ETA(1))
 P = THETA(2)*EXP(ETA(2))
 ALPHA = THETA(3)*EXP(ETA(3))
 L = LA+PEC50
 LL = L*P
 DEN1 = -1*EXP(-2.3026*LL)
 F = ALPHA*EXP(DEN1)
 IPRE = F
 Y = F+EPS(1)
$THETA  7.8 0.84  123
$OMEGA  1 1 .1
$SIGMA 13
$EST MAXEVAL = 2000 PRINT=4 METHOD=1 POSTHOC NOABORT
$COV
$TABLE ID LA E PEC50 P ALPHA IPRE FILE=G_CIRAZ.DAT NOHEADER NOPRINT
```

# Chapter 7

# Summary and conclusions

**Introduction**

This thesis deals with the analysis of repeated measurement data in the field of clinical pharmacology. The repeated assessment in time of subjects under different conditions or treatments introduces a complex structure in the data that may be handled in a number of ways. If interest lies in describing and quantifying the outcome of a study, then often simple summary measures suffice. If the results are meant for extrapolation or for generalisation or for increasing understanding of underlying mechanisms, then mathematical models must be used that allow the data to be placed in a larger knowledge framework.

The preface describes the organisation of the thesis and the first chapter provides a general introduction into analysis methods for continuous repeated measurements. The subsequent chapters describe solutions to practical problems encountered during work at the Centre for Human Drug Research. Several aspects are central to these solutions. First, they aim to extract the maximum amount of information from the data. Second, they deal with identifiability, i.e. whether it is possible to correctly identify certain aspects of the data like treatment response profiles, parameter values or competing models. Third, they provide solutions to the problem of how to deal with missing information on at least some of the individuals.

Maximal information extraction, solving identifiability issues and dealing with missing information often require the combining of information over all subjects. The statistical technique of mixed effect modelling is best suited for this purpose and is applied in most chapters. Linear mixed effect modelling is explained in chapter 2 while chapter 3 deals with the nonlinear version. Mixed effect modelling capitalises on the notion that all subjects are different but have much in common at the same time. Instead of estimating parameters for each subject, average parameters and inter-individual variability estimates are generated. This allows the sharing of information across subjects where missing information of some is supplemented by available information of others.

Although simple summary measures may be preferable for presentation of basic trial outcomes, this thesis shows that models are useful for increasing the understanding of the data. The techniques to analyse the repeated measurement data properly in these situations are investigated and made practically accessible.

**Chapter 1; overview of methods**

The first chapter provides an overview of available methods for the analysis of continuous repeated measurement data.

The most straightforward way to handle repeated measures data is to identify key aspects of the profiles and use these for comparisons. Calculation of these summary characteristics effectively eliminates the repeated measures nature of the data because the sequence of measurements is transformed into a single value. This makes subsequent treatment comparison straightforward because standard statistical tests like the Student's t-test or analysis of variance are usually adequate.

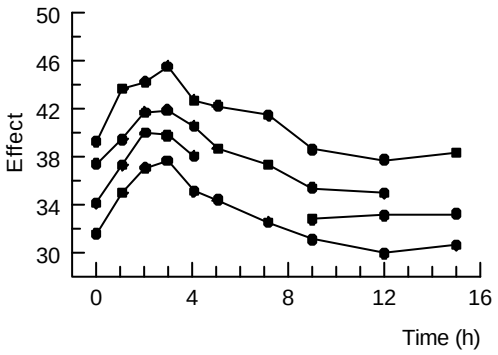
Various model-independent and model-dependent measures are discussed. Model-independent methods are generally best suited to describe study outcome and provide an effective means of compressing the data into easily interpretable characteristics. Examples are measures describing size and time of maximal effect ($C_{max}$ and $T_{max}$), average effect (using means and weighted means in the form of areas under the curve) and time of onset and duration of effect.

Model-dependent methods allow the placing of study results in a larger conceptual framework. If models are available or can be developed that are consistent with the data, then additional and often more general information may be extracted from the data. Well known examples are pharmacokinetic models that describe drug behaviour in terms of useful parameters like volumes, clearances and half-lives. By combining concentrations with effects, parameters related to the time-course, potency and efficacy of the drug may be determined.

Use of summary measures is preferable if allowed by the data. However, if (some of the) individuals provide insufficient information to adequately define the summary measures or if individuals supply estimates with highly different accuracy, then more complex statistical techniques will have to be used. The statistical class of mixed effects models are most useful in this regard. Instead of estimating individual parameters for each subject, the entire data set is analysed collectively but only averages and inter-individual variability of the parameters are estimated. Each individual supplies information according to its relative accuracy. This allows information to be transferred from the individual to the population. Empirical Bayes estimates subsequently allow the population information to be transferred back to the individual by estimating individual parameters conditional on the previously obtained population information.

**Chapter 2; linear mixed effect models**

Lacking an underlying (mechanistic) model to describe the effect profile over time, a plausible assumption may be that each subject has a curve of the same shape but differs from the other subjects by a constant (individual-specific) shift over the entire profile:




Repeated measures analysis of variance (ANOVA) is an appropriate technique to handle these situations. If, however, even a single measurement is missing, the ANOVA model becomes inestimable and the overall treatment profile cannot be identified.

Information on the missing measurements may be borrowed from the remaining subjects by assuming that the missing part for the individual is similar in shape to the other curves with a shift specific to that particular individual.

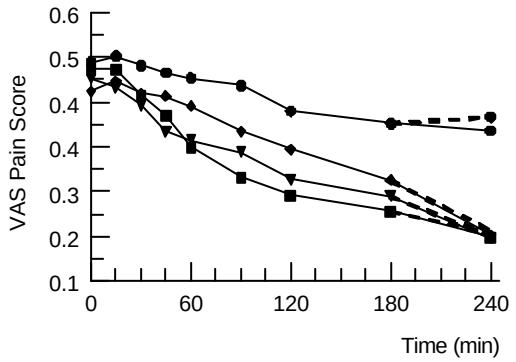
Chapter 2 provides the background to the analysis techniques for complete data in this situation and compares two methods that provide a solution to the missing data problem. Implementation using standard software is described.

The first method simply fills in (imputes) the best estimate for the missing measurement, subsequently followed by a standard repeated measures ANOVA. The second method uses the linear mixed effect model, estimated using restricted maximum likelihood instead of least

squares (the usual ANOVA approach). This allows the model to be fitted to the data even when there are missing measurements. The standard method of significance testing for the mixed effect model is based on large sample arguments which means that the test results are correct if based on an infinite number of measurements. How well this approximation works in practice is unknown.

Simulation results for the two methods are presented in chapter 2. They show that imputation (the first method) leads to falsely rejecting the null hypothesis too often if the number of missing measurements increases. Standard significance testing for the second method also leads to falsely rejecting the null hypothesis too often, which, however, does not seem to be influenced by the amount of missing data. A general small sample correction is proposed and investigated for the second method, leading to adequate type I errors for the simulated design.

If the assumption of similar profiles is justified, then not only completely random missing measurements may be dealt with, but also more elaborate missing data mechanisms. In an analgesia trial for instance, subjects may be allowed to take escape medication if relief is inadequate after a certain amount of time. This means that pain scores after intake of escape medication will be missing. These missing scores, if they had been present, would probably have been higher than the remaining measurements because they are associated with lack of relief. On theoretical grounds, restricted maximum likelihood methodology may still be used to estimate the overall profile under the different treatments even with these missing measurements, if the basic assumptions hold. This would not be possible using simple techniques because the missing measurements would be associated with high pain scores. Averaging the remaining available measurements would result in overestimating the effect of the treatment. This is illustrated with the following graph from a trial investigating the analgesic effects of ibuprofen 200mg (—), ketoprofen 25mg ( – ) and ketoprofen 50 mg ( • ) compared with placebo ( **Ž** ) [1]:



The top line is the placebo response with the largest fraction of escape medication intake. The solid lines give the averages of available cases while the dashed lines provide the linear mixed effect model estimates.

The estimated placebo profile clearly shows that the linear mixed effect model can generate an overall treatment profile that would be expected with the drop out mechanism associated with intake of escape medication. The use of an adequate model makes the identification of the treatment profile possible in the presence of missing data. Basic methods would have failed and analysis would necessarily be restricted to the time points with complete data.

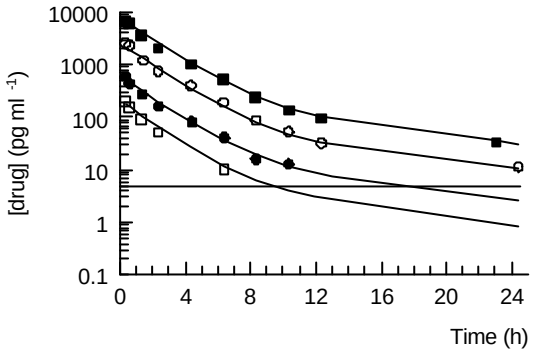
**Chapter 3; nonlinear mixed effect models**

In the practice of clinical pharmacology, mechanistic models are often available to describe the time course of concentrations or effects. The parameters associated with these models are typically estimated using nonlinear regression. Essential pieces of information may, however, be missing for at least some subjects resulting in the inability to identify the model parameters. In these cases, a solution could be to leave the model behind and revert to a more basic presentation of the results. However, if information is combined over subjects using an appropriate statistical technique then parameters may become identifiable after all.

Chapter 3 provides an introduction into the background and use of nonlinear mixed effect models. At the basis lies the deterministic mathematical model describing the profile for a single individual. The link to the actual data is provided by the intra-individual error model that quantifies the deviations between measurements and predictions. All subjects can be estimated simultaneously because the individual subject-specific model parameters are not estimated themselves but rather the population parameters (the mean and variance) of their common distribution. The gain can be illustrated with a study of twenty subjects; if a nonlinear mixed effect model is used then for each model parameter only two population estimates need to be obtained (a mean and a variance) instead of the original twenty individual estimates.

If a subject's profile is incomplete, it can still contribute some information to the overall population parameter estimates. By combining the population information with the individual data, so-called empirical Bayes estimates for each individual can be obtained. If an individual supplies little information, the rest is obtained from the population. This mechanism allows information to be transferred from the population to subjects with incomplete profiles.

An illustration is provided by the first example of chapter 3. In a rising dose study, the terminal part of the curves associated with the initial low doses crosses the detection limit of the assay. This results in an unidentifiable terminal half-life for the low doses. By estimating all doses simultaneously using nonlinear mixed effect modelling, information on the terminal part of the curve that is available for the higher doses may be transferred to the lower doses:




If individual-based methods had been used, the low doses would have been associated with a higher clearance estimate than the high doses because the available information would suggest a smaller area under the curve and a shorter terminal half-life. This could be interpreted as nonlinear pharmacokinetic behaviour that could be of major concern in the development of the drug. However, the dependence of clearance on administered dose can be directly tested in the nonlinear mixed effects model, and no significant relationship is found. This means that the data at this point do not provide an indication of nonlinear pharmacokinetics.

Chapter 3 presents three examples that present parameter identifiability problems where a solution is implemented using nonlinear mixed effect modelling. The first is the previously discussed rising dose study. The second deals with pharmacokinetic parameter estimation of heparin-like substances using what is a pharmacodynamic effect measurement, the anti-Xa activity. As a small amount of anti-Xa activity may be present in the absence of drug, estimation of the terminal part of the curve is often hampered by lingering low activity. By subtraction of non-zero pre-values, the data may all be described by a two-compartment model using nonlinear mixed effect modelling. This solution is analogous to the first example. In the initial publication [2], some curves required a two-compartment model, some were sufficiently described using a one-compartment model and some could not be estimated at all. This illustrates the power of being able to assume a common model for all individuals and subsequently identifying the associated parameters even if the individuals do not provide enough information to allow individual parameter estimation.

An alternative solution to the second example is presented by assuming constant presence of low basal activity. This basal activity is added onto the pharmacokinetic model as a parameter to be estimated. With this solution and using the original data (without pre-value subtraction), a one-compartment model suffices. Interestingly, the two models generate a three-fold difference in clearance estimates; two compartments: 9.44ml/min and one compartment: 32.7 ml/min. Because the two models represent a basically different view, the data themselves cannot be used to determine which of the two models and clearance estimates is correct. This illustrates the point that the model that is used may heavily influence the results, and that additional experiments may be required to provide the final answer. In this case, a multiple dose study will clearly indicate which clearance estimate is best because the

predicted steady state levels differ three-fold for the two models.

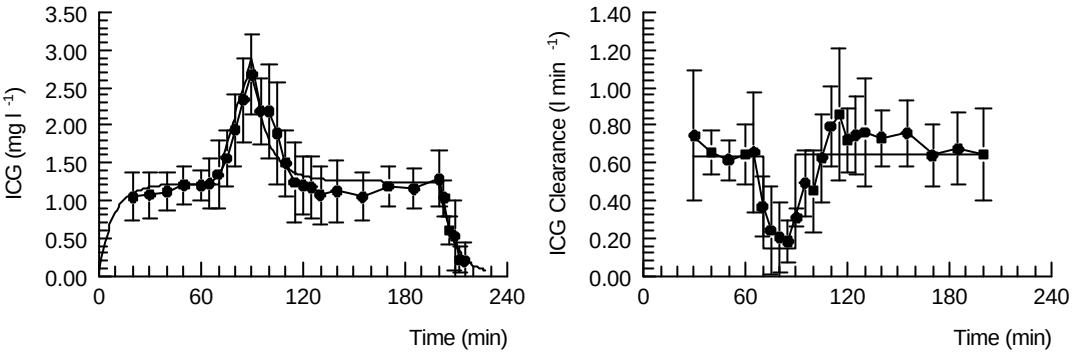
In the third example, pharmacokinetics and pharmacodynamics of heparin-like substances are simultaneously estimated after both intravenous and subcutaneous administration. The model contains so many parameters that accurate estimation requires the combining of information over all individuals. Because both intravenous and subcutaneous information is available, reliable estimates of absorption half-life and bioavailability can be computed. Additionally, the relationship between anti-Xa activity and a general coagulation parameter (the APTT) is estimated.

The examples from chapter 3 illustrate that parameters may be obtained for situations that were formerly thought to be inaccessible to modelling. This approach requires the conviction that all individuals are similar. The same structural model should apply to all subjects, and in the absence of sufficient information, the best parameter estimates for the individual are those that are similar to the estimates of the other subjects. This approach may hide true differences between subjects and may give the appearance of more homogeneity than is actually present. Estimates obtained using nonlinear mixed effect modelling may be the best estimates currently known but they rely heavily on the adequacy of underlying assumptions regarding the structural model and the distribution of the parameters. They can, therefore, never replace the need for well-designed, data-rich studies.

**Chapter 4; identifying clearance profiles**

The clearance of drugs that are rapidly and extensively removed by the liver depends on the rate of blood flow through the liver. Measuring the clearance of such a drug can, therefore, provide an indication of the rate of liver blood flow. If the marker drug is infused continuously, repeated sampling will provide insight into the effect on the marker concentration of clearance changes over time. Chapter 4 deals with the translation of the observed time-concentration profile into the underlying clearance profile. By introducing assumptions about the physiology of the clearance mechanism, this clearance profile may then be translated into a liver blood flow profile. Three different approaches are investigated.

The first approach is only applicable for drugs with single-compartment pharmacokinetics and uses simple estimates of concentration-change to obtain clearance estimates for each time point. Alternative clearance estimates may be obtained using a second nonlinear regression approach, if consecutive periods with constant clearance are assumed. The marker indocyanine green is used to identify the effect of heavy exercise on clearance and liver blood flow using these two techniques. The following graph (left panel) presents measured ICG concentrations (mean"SD) and the estimated average profile using the second nonlinear regression approach:

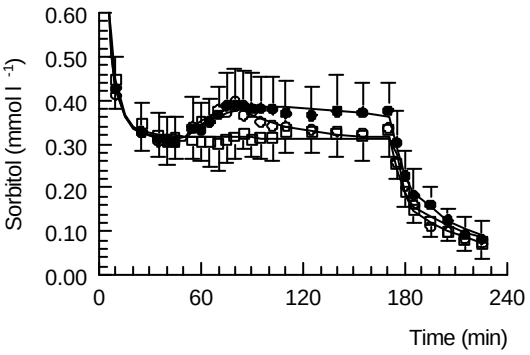



The right panel presents the clearance estimates for each time point (mean±SD) using the first approach and the estimated average clearance profile using the second approach.

The third approach is necessary for drugs with two-compartment pharmacokinetics and requires a parametric description of the clearance profile. Nonlinear regression techniques using the differential equations that define the behaviour of the marker concentration can be used to identify the underlying clearance profile, but only if the basic mathematical form of this clearance profile is given. The regression analysis then provides parameter estimates that define the ultimate shape of the profile. This last approach clearly requires most assumptions that may heavily influence the final parameter estimates.

A range of different profiles may be tested to see which are unlikely and which remain as reasonable options. In this case, sorbitol was used as marker compound and two different intervening drugs with documented effects on liver blood flow (somatostatin and octreotide) were infused in a stepwise manner.

The following graph presents the sorbitol concentrations for the different treatments: octreotide (**Ž**), somatostatin (**F**) and placebo (**G**). The constant rate sorbitol infusion was preceded by a bolus loading dose and ended at 170 minutes, octreotide and somatostatin infusions started at 50min, doubled in rate at 65min and ended at 80 min:




Simple proportionality between intervening expected drug concentrations and changes in

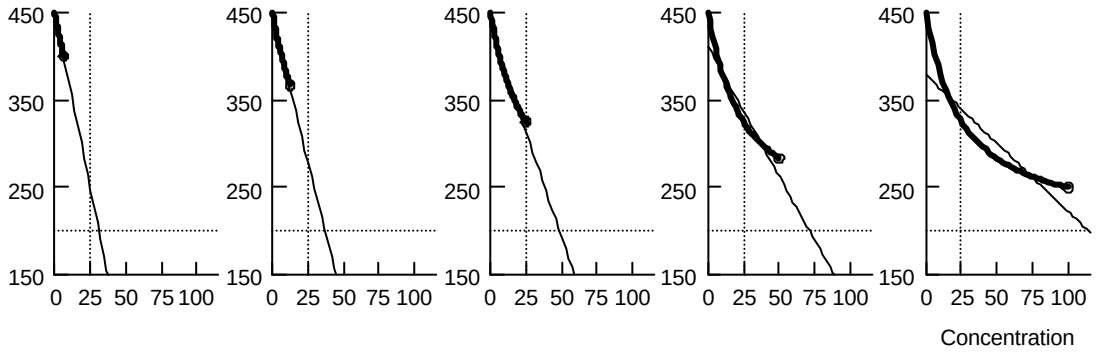
sorbitol clearance could not explain the observed concentration profile. Although the return to a baseline state may be explained by reported half-lives for the intervening drugs, the onset of effect was much more rapid. Although the rate of infusion of somatostatin and octreotide was doubled after 15 minutes, this had very little additional influence on the clearance of sorbitol. This result is corroborated by flows measured in a portal branch using echo-Doppler techniques. The smooth lines in the previous graph provide the average predicted profile for the final model.

The identifiability of the clearance and flow profile and the degree to which information may be extracted from the concentration profile depends to a large extent on the plausibility of the assumptions. The approaches in chapter 4 progress from relatively few to quite a large number of assumptions. While allowing more information to be extracted this also introduces more information in the form of assumptions that may be hard to verify. As always, a balance must be struck and the degree to which the assumptions influence the final statements must be investigated.

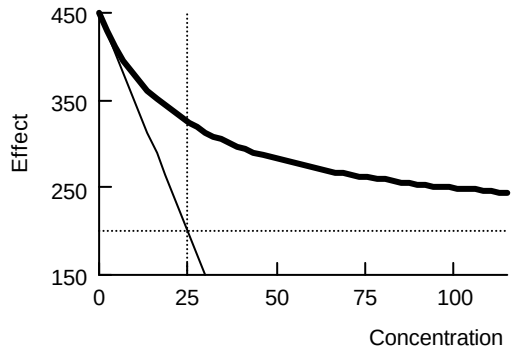
## Chapter 5; comparing incomplete concentration-effect curves

Parameter identification may be difficult in situations where insufficient information is available. Instead of borrowing missing information from other subjects (as in chapters 2 and 3), chapter 5 focuses on means extracting relevant information by re-writing the parameters in the mathematical model.

The $E_{max}$ model is one of the most widely applied mathematical models to describe the relationship between drug concentrations and effects. The most important model parameters, $E_{max}$ and $EC_{50}$ can only be determined with reasonable precision if the observed data shows asymptotic behaviour consistent with approaching $E_{max}$. If this is not the case then a wide range of values may be used to describe the same curve. If only a small initial part of the $E_{max}$ curve is available then ordinary linear regression could be used to estimate drug sensitivity. However, if more of the curve becomes available, the estimated straight line will tend to flatten out as illustrated in the next graph:

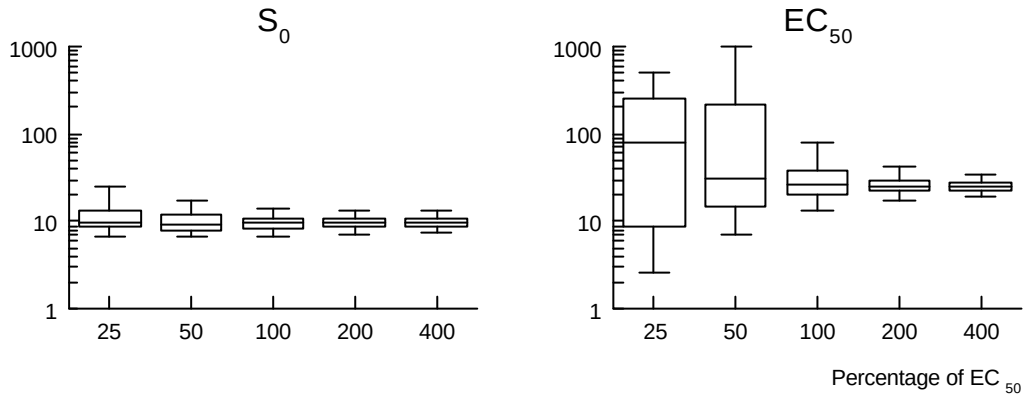



This means that a simple straight line provides an unacceptable solution. If the $E_{max}$ model is used in the situation as described in the above graph, then $E_{max}$ and $EC_{50}$ are very poorly estimated, as shown in the left hand panels. The ratio of $E_{max}$ to $EC_{50}$ is much less variable however, even in the severely truncated curves. This ratio is equal to the slope of the tangent to the $E_{max}$ curve at zero concentration and is almost equal to the slope of the straight line fitted to concentration-effect data that have their maximum far below $EC_{50}$:



By rewriting the $E_{max}$ equation in terms of $E_{max}$ and $S_0$ (which equals the ratio of $E_{max}$ to $EC_{50}$) both situations with incomplete curves and with full curves can be described.

When comparing drugs or when comparing different doses of the same drug, often only a fraction of the entire $E_{max}$ curve is covered by the data. Simulations in chapter 5 indicate that in these cases, use of $S_0$ as a measure of drug-sensitivity is superior to the use of $EC_{50}$, being estimated with far greater precision. Five levels of truncation were simulated with 100 curves each. The level of truncation is indicated by the maximum concentration reached, as a percentage of $EC_{50}$. The following graph presents the result for the $S_0$ and $EC_{50}$ estimates of one of eight simulations; the box encompasses 50% of the estimates, the whiskers indicate the 5% and 95% percentile of the estimates, and the median is represented by the horizontal line in the box:




The $S_0$ parameter is even superior to $EC_{50}$ when the curve approaches 100% $E_{max}$, making it a sensible alternative parameter in any situation.

Two examples in chapter 5 illustrate the use of the $S_0$ parameter to identify changes in sensitivity in a rising dose design and to detect differences in sensitivity between a new drug and an active comparator. The examples are from two rising dose studies [3,4] that investigate the sedative effects of new benzodiazepines compared to midazolam. The drugs were infused over 20 minutes or until conscious sedation was reached.

In the original publications, differences in sensitivity between the new drug and midazolam had to be compared on the basis of the plasma concentrations measured at the point of reaching conscious sedation. This meant that only the high dose levels could be used and comparison was based on only a single point on the concentration-effect curve. Average and individual concentration-effect graphs for one of the drugs (Ro 48-8684) suggested a decrease in sensitivity with increasing dose, which could not be tested formally.

Reanalysis of the studies (described in chapter 5) using the $S_0$ concept indicated a significant 58% decrease in $S_0$ and therefore in sensitivity (95%CI: 7.5%/81%) when the 1mg dose of Ro 48-8684 was compared to the 10mg dose. This was not detected for Ro 48-6791 (decrease: 41%; 95%CI: !215%/84%). Both drugs were more potent than midazolam; midazolam sensitivity was 50% lower (95%CI: 5.6%/74%) when compared to Ro 48-8684 and 93% lower (95%CI: 76%/98%) when compared to Ro 48-6791.

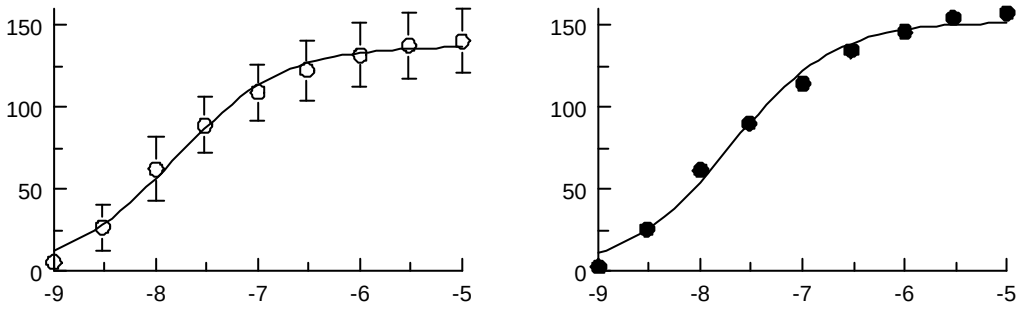
Use of $S_0$ enables quantification and comparison of sensitivity to drug effects where only a qualitative comparison was possible using basic methods.

**Chapter 6; model identification**

Practice often dictates a choice between competing models. A simple model may indicate a systematic pattern in the deviations of observed from predicted values. Increase of model-complexity generally results in a better description of the data but significance tests are required to prove that the increase in explained variability is not due to chance alone.

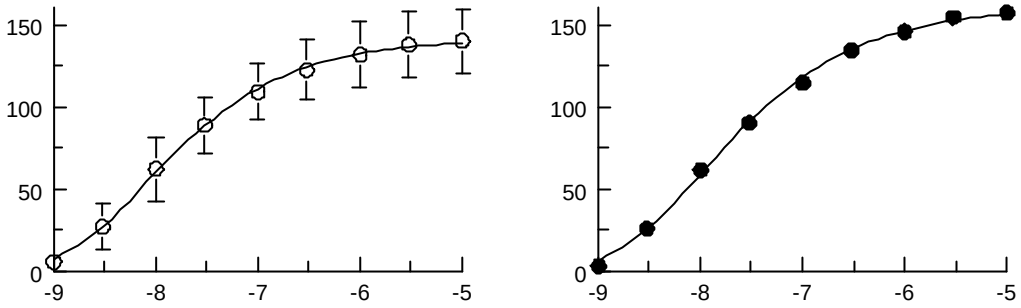
If one model may be seen as a simplification or restriction of the other then likelihood ratio tests may be used to test for significance. Using standard nonlinear regression this implies a separate significance test for each individual. If the data set is analysed collectively using nonlinear mixed effect modelling then a single model comparison may be performed for the entire data set.

The logistic model is widely used in *in-vitro* pharmacology to describe the relationship between the logarithm of the concentration and the effect. It is similar to the sigmoid $E_{max}$ model and one can be rewritten in terms of the other. Chapter 6 describes a class of drugs ($á_1$-adrenoceptor agonists) and their effect on rat-aorta segments. Small but consistent and systematic deviations are seen when using the logistic model as illustrated in the next graph:




The right panel indicates the fit for an individual experiment and the left panel the average ("SD) fit for all experiments.

An alternative model is suggested that is not symmetric like the logistic model. When the data are fitted to this asymmetric Gompertz model, the deviations decrease as illustrated in the next graph:




The Gompertz model cannot however be expressed as a (restricted) logistic model or vice versa. The two competing models have the same number of parameters and for these reasons likelihood ratio testing cannot be used to determine whether one model is significantly better than the other. There is however a 'super model' (the Richards model) which has both the logistic and the Gompertz as sub-models. The Richards model has very poor parameter estimating properties. Parameter estimation for individuals separately is practically impossible, but collective estimation using NONMEM allows adequate determination of parameters and opens up the perspective of a single comparison of the sub-models to the super-model. For most of the investigated drugs this resulted in an indistinguishable fit for the Richards and the Gompertz model and a highly significant difference between the logistic and the Richards model. This clearly indicates the superiority of the Gompertz model.

The asymmetry in the concentration-effect curves is attributed to heterogeneity in the receptor population. Interestingly, blocking a receptor sub-population results in a concentration-effect curve that is better described using the logistic model than the Gompertz model.

The use of a super model may be an exceptional solution to a model comparison problem but often models can be seen as extensions of more basic models. One-compartment and two-compartment pharmacokinetic models may be compared. Linear, $E_{max}$, and sigmoid $E_{max}$ concentration-effect model may be compared, and it is possible to test whether inclusion of a hypothetical effect-compartment in pharmacokinetic/pharmacodynamic modelling results in a significant improvement in fit. By using nonlinear mixed effect modelling in all these cases, a single unambiguous statement may be issued regarding superiority of one model over another.

## Conclusions

The classification of analysis methods for repeated measurement data can be made along two axes. On the one axis there is the contrast between summary measures and analysis of the entire data set as a whole. On the other axis there is the contrast between model-independent and model-dependent methods.

As has been argued in chapter 1, the choice for summary measures is preferable if the data support it. The fact that the remainder of the thesis is almost entirely devoted to analyses of entire data sets as a whole, illustrates that this ideal situation is not always present. Fortunately, statistical mixed effects models allow the extraction of characteristics from the data in these less than ideal situations.

The need to deviate from simple summary measures is almost invariably dictated by the absence of sufficient information for at least some of the individuals. This may be caused by missing data (either by misfortune or by design) or by the incomplete characterisation of the underlying process by the data. The main and motivating advantage for the use of mixed effects models is the gathering of strength. The first step from individual to population comes about by combining individual information of variable quality and accuracy into a single population estimate. This way, all relevant available information (however limited) may be used. In the second step, empirical Bayes estimates allow the transfer of population information back to the individual, without ignoring the (possibly limited) information that is present in the data for the individual. This movement from individual to population and back allows the sharing of information between individuals, while providing the best possible estimate for the population under investigation.

The choice between model-independent and model-dependent measures is largely dictated by the objectives of the investigation and often a combination of both is used. The split between the two methods is largely parallel to the distinction between 'confirm' and 'learn' studies [5]. If a study is designed to confirm pre-existing notions, the type and size of the response is often fairly well known. In this cases the test for presence or absence of differences can be operationalised by pre-defining summary measures on the basis of which decisions can be made. By keeping these measures as simple as possible, the discussion of study outcome will be focussed on the objectives of the study, without being side-tracked by discussions about the means of quantifying the study results.

If on the other hand the study is designed to learn rather than to confirm, this learning process may be enhanced if a mathematical model can be found or developed that is consistent with the data. Naturally, the results may be initially summarised by model-independent summary measures, but by estimating the relevant model parameters it may be easier to generalise the information obtained from the study. Estimation of pharmacokinetic parameters for instance may allow the prediction of concentration profiles for input regimes that were not initially studied.

By translating (qualitative) physiological information into a mathematical model, it becomes possible to test whether what we think we know is supported by the data. Mathematical model building is always an iterative process with a continuous going back and forth from the model to the data. If model-building is successful then the language of mathematics is used to build a structure for the knowledge about the process or system studied. Statistics links theory to reality by providing a means of testing whether the mathematical construct is consistent with the data as observed in the real world.

The field of clinical pharmacology is uniquely suited to the development of models for drug action. By providing the methods to link concepts and ideas to real data, the statistician, with one foot in the field of biology and medicine and the other in the field of mathematics and statistics, will be a builder of bridges from theory to reality and back.

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# Summary in Dutch;
# Nederlandstalige samenvatting

## Het modelleren van herhaalde metingen binnen de klinische farmacologie; van individu naar populatie en terug

Klinisch farmacologische experimenten onderzoeken het effect en concentratie-verloop van geneesmiddelen bij de mens. In de praktijk betekent dit meestal dat op een aantal tijdstippen na elkaar, bloed wordt afgenomen en maten voor de werkzaamheid van het geneesmiddel worden bepaald. Zo zal voor een middel tegen hoge bloeddruk op vaste tijden een bloeddruk meting herhaald worden. Dit leidt tot een reeks van metingen met bijbehorende tijdstippen. De analyse van zo'n reeks van herhaalde metingen waarbij gebruik gemaakt wordt van modellen, is het onderwerp van dit proefschrift.

Modellen geven een wiskundige beschrijving van de relatie tussen datgene wat gemeten wordt zoals concentratie of effect, en datgene wat van invloed is op de hoogte van die meting zoals tijdstip van afname of toegediende dosis. Een farmacokinetisch model bijvoorbeeld beschrijft een curve van geneesmiddel concentratie in de tijd, waarbij de vorm van de model curve afhankelijk is van de waarde van zogenaamde model parameters. Met behulp van regressie methoden wordt de waarde van de parameters zo gekozen dat de resulterende curve het best past bij de gevonden metingen. De wiskunde onderscheidt lineaire en niet-lineaire modellen waarbij de model parameters voor lineaire modellen het meest eenvoudig te schatten zijn. Bij niet-lineaire modellen zijn geen simpele formules te geven om de best passende model parameter te bepalen aan de hand van de metingen. Modellen die ontwikkeld zijn op basis van ideeën over biologische werkingsmechanismen (en die daardoor het meest interessant zijn) zijn bijna altijd niet-lineair.

De meest eenvoudige manier om herhaalde metingen te analyseren, is door de reeks getallen terug te brengen tot één enkel getal dat een aspect van de reeks samenvat. Uit het tijdsverloop van de bloeddruk kan bijvoorbeeld de gemiddelde bloeddruk of de laagst

gemeten bloeddruk worden bepaald. Ook kan met behulp van een model de relatie beschreven worden tussen geneesmiddel concentratie en bloeddruk daling. Eén van de model parameters levert dan bijvoorbeeld een maat voor de werkzaamheid van het geneesmiddel

Het grote voordeel van zo¤n samenvattende maat is dat het herhaalde-metingen aspect verdwijnt. Bij het onderzoeken van twee middelen tegen hoge bloeddruk hoeft men slechts een enkel getal te vergelijken (de minimaal bereikte bloeddruk bijvoorbeeld) in plaats van de complete reeks van metingen. De benodigde statistische methoden kunnen dan ook eenvoudig blijven, wat deze techniek in de praktijk erg aantrekkelijk maakt.

Problemen ontstaan echter op het moment dat de reeks onvoldoende informatie bevat om een redelijke schatting van zo¤n samenvattende maat te maken. Als er bijvoorbeeld metingen ontbreken is het niet goed mogelijk om een gemiddelde te bepalen. Een oplossing kan verkregen worden als de ontbrekende informatie aangevuld zou kunnen worden. Die aanvulling kan komen van individuen die wèl een complete reeks metingen hebben.

De statistische methode van de *mixed effect modelling* leent zich bij uitstek voor het delen van informatie tussen individuen. Niet langer wordt voor elk individu afzonderlijk een schatting voor de samenvattende maat berekend, maar alle individuen worden tegelijkertijd in één groot model geanalyseerd. Voor elke samenvattende maat wordt alleen een gemiddelde voor alle individuen en een maat voor de verschillen tussen individuen berekend. Op deze manier kan elk individu bijdragen aan het totaal, hoe beperkt de eigen bijdrage ook mag zijn.

*Mixed effect modelling* maakt de overdracht van informatie van individu naar populatie mogelijk. De verkregen populatie-informatie kan vervolgens weer teruggebracht worden naar de individuen door gebruik te maken van *empirical Bayes estimates*. Dit zijn schattingen voor het individu, waarbij gebruik wordt gemaakt van de eerder verkregen populatie informatie. Een samenvattende maat voor een individu met veel ontbrekende informatie wordt dan voor een groot gedeelte bepaald door de populatie informatie. Een individu met veel eigen informatie zal weinig hoeven te lenen. Op deze manier kan informatie effectief gedeeld worden tussen de individuen.

Het is niet altijd nodig om modellen te gebruiken bij de analyse van herhaalde metingen. De gemiddelde of minimale bloeddruk waarde is een voorbeeld van een zogenaamde model-onafhankelijke maat. De model-onafhankelijke methoden die geen gebruik maken van modellen, hebben als voordeel dat ze eenvoudiger zijn en gemakkelijker te begrijpen. Dit maakt deze methoden in de praktijk aantrekkelijk.

Door wèl modellen te gebruiken kan meer informatie uit de data worden verkregen en bestaat de mogelijkheid om de gegevens in een groter geheel te plaatsen. Met behulp van een model-afhankelijke maat voor de werkzaamheid van een geneesmiddel, kan bijvoorbeeld een voorspelling worden gegeven over het effect als de helft van de dosis wordt gegeven. Tegelijkertijd kunnen problemen opgelost worden die te maken hebben met onvolledige informatie bij een deel van de individuen; *mixed effect modelling* kan niet zonder modellen.

Alle in het proefschrift beschreven oplossingen maken gebruik van modellen om zoveel

mogelijk informatie uit de data te halen. Bijna alle oplossingen maken gebruik van *mixed effect modelling* om problemen met onvoldoende informatie aan te pakken.

*Hoofdstuk 1* geeft allereerst een overzicht van verschillende methoden voor de analyse van herhaalde metingen. Vervolgens beschrijft dit proefschrift een aantal situaties waarin modellen gebruikt zijn bij de analyse van herhaalde metingen. Praktische oplossingen worden gegeven voor analyse problemen zoals die zich hebben voorgedaan op het Centre for Human Drug Research.

*Hoofdstuk 2* beschrijft de analyse van studies waarbij elke proefpersoon een aantal behandelingen ondergaat en bij elke behandeling een aantal metingen worden gedaan. Bij gebrek aan een geschikt model om de metingen te beschrijven kan gekeken worden naar het gemiddelde effect op elk afzonderlijk tijdstip. Als deze gemiddelden grafisch uitgezet worden tegen de bijbehorende tijdstippen kan het zo verkregen profiel een indruk geven van het effect verloop in de tijd. Zo▫n gemiddeld profiel kan volstaan, als de individuele profielen tenminste voldoende op elkaar lijken.

Dit soort gegevens wordt vaak geanalyseerd met behulp van variantie analyse, maar daarvoor mag geen enkele meting ontbreken. In hoofdstuk 2 worden twee oplossingen onderzocht als er toch metingen ontbreken: het invullen van een passend getal op de open plek, of het analyseren van de metingen gebruik makend van een *linear mixed effect model*.

Bij een goede statistische methode is van tevoren bekend hoe groot de kans is op de onjuiste uitspraak dat de onderzochte maten verschillen terwijl ze dat in werkelijkheid niet doen. Deze kans op een zogenaamde type I fout wordt meestal gesteld op 5%.

Beide in hoofdstuk 2 onderzochte methoden blijken een te grote kans te hebben op een type I fout. De invulmethode wordt daarbij steeds slechter naarmate er meer data ontbreken. Het *linear mixed effect model* geeft standaard een te grote kans op een type I fout, onafhankelijk van de hoeveelheid ontbrekende gegevens. Het probleem met de invulmethode valt niet gemakkelijk op te lossen, maar voor het *linear mixed effect model* wordt in hoofdstuk 2 een eenvoudige correctie voorgesteld. In het onderzochte geval leidt de correctie er toe dat de kans op een type I fout weer de verwachte 5% wordt.

*Hoofdstuk 3* beschrijft een oplossing voor het bepalen van parameters van niet-lineaire modellen als de individuen zelf te weinig informatie leveren om tot goede schattingen te leiden. Door informatie te delen, gebruik makend van *nonlinear mixed effect modelling,* kunnen de gegevens als één geheel worden gepresenteerd. Parameters kunnen geschat worden waar dat voorheen onmogelijk was. In één van de voorbeelden worden kinetische parameters berekend (zoals de halfwaardetijd) in een proefopzet waarbij opklimmende doses van een nieuw geneesmiddel worden toegediend. Bij de lagere doseringen verdwijnt het laatste gedeelte van de concentratie-tijd curve onder de detectie-limiet van de bepalingsmethode. Hierdoor kan de laatste fase van de curve niet beschreven worden. Door de informatie van hogere doseringen te gebruiken kunnen zinvolle parameter schattingen worden verkregen. Bovendien is het mogelijk om te kijken of er aanwijzingen zijn of de kinetiek afhangt van de toegediende dosis; bij geneesmiddel ontwikkeling zal men dat in een zo vroeg mogelijk stadium willen weten.

*Hoofdstuk 4* beschrijft kinetische methoden die een indruk kunnen geven van de snelheid waarmee bloed door de lever stroomt. Dit is belangrijk omdat voor een aantal stoffen geldt dat de snelheid waarmee ze uit het lichaam verdwijnen voornamelijk afhangt van de snelheid waarmee ze aangeboden worden aan de lever. Door een continu infuus van zo'n stof aan te leggen en op geregelde tijden monsters te nemen, kunnen de gevolgen van een verandering in leverstroom-snelheid onderzocht worden. Bij een daling van de leverstroomsnelheid (bijvoorbeeld doordat de proefpersoon moet fietsen) wordt de stof slechter verwijderd zodat de concentratie stijgt. De gemeten concentraties lopen echter altijd achter bij de veranderingen in stroomsnelheid omdat het even duurt voordat een nieuw evenwicht is ingesteld.

Het vertalen van die concentraties in de onderliggende snelheden wordt beschreven voor twee verschillende model stoffen: indocyanine groen (ICG) en sorbitol. Voor ICG gaat dat wat eenvoudiger dan voor sorbitol, omdat de kinetische eigenschappen van ICG eenvoudiger zijn. De methode is voor sorbitol dan wel ingewikkelder maar levert wel meer informatie op. Niet alleen kan de mate van verandering in leverstroomsnelheid vastgesteld worden, ook de absolute hoogte (in liter per minuut) kan nauwkeurig worden bepaald. ICG is eigenlijk alleen geschikt voor het vaststellen van de mate van verandering, uitgedrukt als percentage daling of stijging.

*Hoofdstuk 5* geeft een oplossing voor een veel voorkomend probleem bij het vastleggen van de relatie tussen concentraties van geneesmiddelen en de tegelijkertijd gemeten effecten. Vaak blijkt dat een verdubbeling in concentraties niet leidt tot een verdubbeling van effecten omdat het effect aan een maximum is gebonden. Zo'n relatie tussen concentratie en effect wordt vaak beschreven met het zogenaamde $E_{max}$ model. Het blijkt in de praktijk dat het maximum vaak bij lange na niet bereikt wordt, bijvoorbeeld omdat dit gepaard gaat met allerlei ongewenste bijverschijnselen. De model parameters van het $E_{max}$ model kunnen echter alleen goed geschat worden als het maximum ook daadwerkelijk bereikt wordt. Vooral de maat die de gevoeligheid van het effect karakteriseert (de $EC_{50}$ die de concentratie geeft waarop 50% van het maximale effect optreedt) is erg slecht te bepalen als maar een deel van de curve aanwezig is.

Een oplossing wordt gegeven in hoofdstuk 5 door een alternatieve gevoeligheids-maat te gebruiken. Deze nieuwe gevoeligheidsmaat ($S_0$ gedoopt) geeft de steilheid van de curve bij lage concentraties weer. Met behulp van deze $S_0$ wordt de vorm van de concentratie-effect relatie net zo goed beschreven als met de $EC_{50}$. Maar de $S_0$ kan veel nauwkeuriger bepaald worden dan de $EC_{50}$ bij curven die hun maximum bij lange na niet bereiken. Doordat gevoeligheid nauwkeuriger vastgesteld kan worden, wordt het mogelijk om te onderzoeken of die gevoeligheid voor een geneesmiddel verandert naarmate er meer van wordt toegediend. Ook kan de gevoeligheid voor verschillende geneesmiddelen goed met elkaar vergeleken worden.

*Hoofdstuk 6* tot slot, beschrijft een oplossing voor het kiezen uit twee concurrerende modellen. De relatie tussen concentraties en effecten in laboratorium-omstandigheden wordt vaak beschreven met behulp van het zogenaamde logistische model. De door dit model voorspelde curve is symmetrisch ten opzichte van de $EC_{50}$. In de praktijk blijkt dat sommige stoffen systematische afwijkingen ten opzichte van die symmetrische curven laten zien. Dit zou een aanwijzing kunnen zijn voor de aanwezigheid van verschillende receptoren.

De asymmetrische curven lijken beter beschreven te worden met het zogenaamde Gompertz model. Klassieke toetsingstheorie kan alleen modellen vergelijken waarbij het ene model te herleiden is uit het andere model door sommige parameters constant te houden. Het Gompertz en het logistische model lijken niet op elkaar en hebben bovendien allebei evenveel parameters zodat de toetsingstheorieën niet toepasbaar zijn.

De oplossing ligt in een nieuw model dat beide oude modellen tot subtype heeft. Zo'n model bestaat en heet het Richards model. De parameters van dit model zijn alleen erg slecht te schatten. Door gebruik te maken van *nonlinear mixed effect modelling* kunnen toch betrouwbare schattingen worden verkregen door de gegevens van de verschillende experimenten te combineren.

Het Gompertz model blijkt in bijna alle gevallen veel beter te passen. De enige uitzondering vormt een experiment waarbij een deel van de receptorpopulatie uitgeschakeld is. De overgang van een asymmetrische naar een symmetrische curve zou er op kunnen wijzen dat er maar één type receptor is overgebleven.

De verschillende verhalen uit dit proefschrift laten zien dat door het gebruik van modellen en het combineren van informatie, oplossingen kunnen worden gevonden voor belangrijke analyse problemen in de (klinische) farmacologie. De statisticus levert daarbij het materiaal om een brug te kunnen bouwen tussen de metingen en de (klinisch farmacologische) theorie.

# Curriculum Vitae

Hendrik Cornelis (Rik) Schoemaker was born March 24, 1963 in Amsterdam (Netherlands) but has lived practically all his life in Amstelveen. The one notable exception occurred from the summer of 1975 until the summer of 1977 when he visited Baltimore (Maryland, USA) and attended the 7th and 8th grade of Dumbarton Junior High School. In 1982 he obtained his Atheneum-B diploma at the Hermann Wesselink College in Amstelveen and started his biology study at the Free University of Amsterdam. He graduated cum laude in 1987 with a specialisation in mathematical biology (model building, methodology and statistics). Subsequently, he served as conscientious objector to the military service as mathematical modeller and statistician at the Polyclinic for Reproductive Endocrinology and Fertility Research (VEVO, Free University Hospital, Amsterdam). Since March 1989 he holds a position as statistician at the Centre for Human Drug Research[1].

Aside from the four publications as first author presented in this thesis, he is co-author of over 70 international publications, demonstrating his wide involvement as statistician/ pharmacokineticist/mathematical modeller.

He is the partner of Marieke de Kam, and proud father of his two sons Linus and Maxim and his daughter Febe.

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# Nawoord

Altijd heb ik de stelling onderschreven dat plezier in je werk voor het overgrote deel bepaald wordt door de mensen waar je mee samenwerkt. Wat dat betreft heb ik de afgelopen tien jaar op het CHDR geen enkele reden tot klagen gehad. De vele mensen die ik daar heb leren kennen zorgden ervoor dat ik bijna altijd met plezier naar mijn werk ging.

Ik denk daarbij aan mijn baas/begeleider, mijn direkte collega's (zowel binnen als buiten het CHDR) en de vele anderen waarmee ik koffie (met warme melk), taart, boterhammen, te slappe thee en heel veel verhalen en belevenissen heb gedeeld. Dank jullie wel!

Laat het werk zelf nou ook nog leuk zijn....