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Identification of specific intrinsic liver clearance from a precision-cut liver-slice experiment

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Abstract

A model describing metabolism experiments with precision-cut liver-slices incubated in a culture medium is developed. Formal mathematical techniques are presented that solve the problem of identifying the specific intrinsic liver clearance from the experimental data. The formal solution is discussed from the perspective of experimental practice. A necessary condition for identification is sampling parent compound in slice or culture medium. However, sampling parent compound in slice and additionally metabolite pooled from medium and slice is required by experimental limitations. Moreover it appears that identification is unreliable when the value of the intrinsic clearance of the slice exceeds the value of a diffusion parameter describing transport of the parent compound from the culture medium to the slice, a condition to be verified only afterwards.

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Samenvatting

In vitro experimenten met zeer dunne plakjes lever (liver-slices) worden uitgevoerd ten behoeve van de identificatie van de snelheid van leverklaring van stoffen. Het blijkt dat experimentele resultaten voorzichtig geïnterpreteerd moeten worden.

Een fysisch-chemisch model voor dit soort experimenten wordt geïntroduceerd, tezamen met een stel model parameters. Dit model leidt tot een wiskundig modelsysteem met bijbehorende systeemparameters die zijn uitgedrukt in de modelparameters. De analytische oplossing van het modelsysteem is uitgedrukt in termen van observabele parameters, d.w.z. parameters die kunnen worden geïdentificeerd uit het experiment. Deze observabele parameters op hun beurt zijn uitgedrukt in termen van de systeemparameters. Er zijn maar 3 observabele parameters en ook maar 3 systeemparameters. Om de klaring te kunnen identificeren uit het experiment is kennis vooraf van een aantal modelparameters nodig. Zo blijkt bijvoorbeeld dat het vereist is om de niet gebonden fractie van de stof in kweekmedium te kennen. Ook blijkt dat de klaring alleen geschat kan worden uit de experimentele data door tegelijkertijd de vrije fractie van de stof in de slice en een coëfficiënt die de diffusie in het model beschrijft uit de data te schatten.

Één van de motivaties van dit onderzoek is de toepassing van kinetische gegevens in PBPK modellen. Daarom wordt aangegeven hoe de klaring verkregen uit het slice-experiment, moet worden geëxtrapoleerd van de *in vitro* (slice) naar de *in vivo* (totale lever) situatie. Bij deze extrapolatie blijkt dat ook de vrije fractie van de stof in lever bekend moet zijn, maar die moet al mede uit de data geïdentificeerd worden om tot identificatie van de klaring te komen. Een van de bijkomende vereisten voor extrapolatie is dat de niet gebonden fractie van de stof in bloed bekend moet zijn, in analogie met de benodigde kennis van de vrije fractie van de stof in kweekmedium voor het *in vitro* experiment.

Formele wiskundige technieken worden toegepast op het modelsysteem om het identificatieprobleem voor de intrinsieke leverklaring op te lossen, d.w.z. welke grootheid of grootheden bemonsterd moeten worden om uit de experimentele data de klaring en de vrije fractie te identificeren. Hieruit blijkt dat het op zijn minst nodig is om de hoeveelheid moederstof in slice of kweekmedium afzondelijk te bemonsteren. Bemonstering van de hoeveelheid moederstof of de hoeveelheid metaboliet in slice en kweekmedium gezamenlijk is onvoldoende voor identificatie.

De formele wiskundige oplossing wordt getoetst aan de gesimuleerde praktijk. Daarbij wordt er vanuit gegaan dat de tijdsduur van een experiment beperkt is (1 uur), dat er een beperkt aantal bemonsteringen wordt gedaan en dat de data willekeurige fouten hebben tengevolge van analysefouten en het feit dat voor ieder datapunt met andere slices wordt gewerkt. Deze meer practische benadering van het identificatieprobleem leidt tot aanvullende aanbevelingen m.b.t. de experimentele opzet: bemonstering van de hoeveelheid metaboliet in slice en medium gezamenlijk is dan wel vereist voor identificatie.

Uit een gevoeligheids- en foutenvoortplantingsanalyse blijkt tevens dat het schatten uit de experimentele data van de klaring en de vrije fractie tegelijkertijd onbetrouwbare resultaten oplevert indien de waarde van de klaringsparameter groter wordt dan de waarde van de diffusieparameter, die ook een van de te schatten modelparameters is.

Summary

In vitro experiments with precision-cut liver slices are performed for the identification of the metabolism rate of compounds. It appears that one should be careful in interpreting experimental results.

A physico-chemical model describing the slice experiment is introduced, together with a set of *model parameters*. From this model a mathematical system model is derived. The set of *system parameters* of this last model are in terms of the underlying model parameters. The analytical solution to the system model is expressed in terms of *observable parameters*. These are parameters that can be identified from the experiment. These observable parameters, in turn, are expressed in terms of the system parameters. There are only three observable parameters at maximum, so for identification purposes *a priori* knowledge of a number of model parameters is required. E.g., it appears that it is required to know the free fraction of the compound in culture medium. Also, it appears that clearance can only be identified by simultaneously identifying the compound's free fraction in the liver slice and a coefficient describing diffusion of the compound.

One of the motivations of this research is the application of kinetic data in PBPK modelling of *in vivo* systems. Therefore, it will be outlined how to extrapolate results from the *in vitro* slice model to a PBPK model describing the liver *in vivo*. Extrapolation requires the free fraction of the compound in the liver, which is estimated from the *in vitro* experiment. Also the free fraction of the compound in blood is required for extrapolation purposes, analogous to the requirement of knowledge of the free fraction of the compound in culture medium.

Formal mathematical techniques are applied to the system model solving the identification problem for specific intrinsic liver clearance. From this analysis one obtains minimal requirements as to what data should be sampled for identification of the unknown parameters. From this analysis it appears that at least the amount of parent compound in slice or culture medium (not pooled) should be sampled. Sampling metabolite, pooled or not, from culture medium and slice, or sampling the pooled parent compound is insufficient for identification.

The theoretical considerations will be challenged by simulating experiments, i.e., obtaining results from the analytical solution perturbed by random errors and within a limited time of 1 hour interval only. This more practical consideration of the identification problem will lead to recommendations of experimental set-up additional to the formal requirements. E.g., the additional sampling of pooled metabolite is required in these circumstances.

Moreover, from a sensitivity and error propagation analysis it appears that the simultaneous identification of clearance and free fraction of the compound produces unreliable results whenever the clearance exceeds diffusion, which is also one of the parameters to be identified from the experiment.

1. Introduction

In vitro liver-slice experiments may be performed to identify the metabolism rate of a drug (Worboys *et al.*, 1995; Worboys *et al.*, 1996; Worboys *et al.*, 1997) or environmental contaminant. For our purposes, these rate constants are requisite in modelling the *in vivo* kinetics of a compound. One should be careful in interpreting the results from a slice experiment. Besides metabolism, other processes, such as protein binding or sequestration in the lipid fraction of the slice and diffusion of compounds between culture medium and slice, are involved. They should be considered in analysing experimental results.

Worboys *et al*. (1997) assessed drug metabolism in rat liver slices by analysing experimental data using a classical one-compartment model. This model describes slice uptake as an apparent exponential increase to a plateau value. However, as the experimental model consists of two physically different phases, slice and culture medium, a two-compartment model at least seems to be more appropriate.

In this report, a mathematical model describing the slice experimental model will be presented. This mathematical model is, like for instance PBPK models of *in vivo* systems, based on the underlying processes of transport, partitioning and elimination. For clarity of presentation a compound with only one metabolic pathway, having no secondary metabolites will be considered. However, the results in the report can still be applied when secondary metabolites are formed. In this case one should read "the total of metabolites ", i.e. the primary, secondary, ternary (and so on) metabolites, for the term "metabolite": e.g., sampling metabolite should be interpreted as sampling the primary, secondary, ternary (and so on) metabolite. Moreover, when there are several pathways the term "metabolite" also should be interpreted as "the total of metabolites". In this case the metabolism rate is the sum of the rates for the different pathways.

One of the motivations of this research is the application of kinetic data in PBPK modelling of *in vivo* systems. Therefore, it will be outlined how to extrapolate from the mathematical model, describing the slice experimental model, to a PBPK model, describing the liver *in vivo*. Extrapolation is based on the underlying processes of transport, partitioning and metabolism in both models.

The mathematical model will also be applied for obtaining the solution to the identification problem, i.e., whether the required parameters can be obtained from experimental data, given a typical experimental set-up, or not. From a formal analysis of the mathematical model together with a formalised representation of the experimental set-up, it can be decided whether theoretically the metabolic rate can be identified from an experiment.

The theoretical considerations will be challenged. This is achieved by simulating experiments, i.e., obtaining results from the analytical model solution perturbed by random errors and within a limited time interval only. This more practical consideration of the identification problem will lead to additional recommendations for the experimental set-up in order to yield parameter identification in practice. Moreover, conditions are recognised that indicate whether the solution to the identification problem is reliable or not.

2. Slice experiment models

2.1 Physico-chemical model

During a typical *in vitro* experiment a liver slice is incubated in culture medium containing the compound to be investigated with initial concentration $C_{P,m,0}$ (*'P'* for parent compound, *'m'* for culture medium; see figure 1, top). The compound diffuses from culture medium into the liver slice. This process can be divided into 3 sub-processes: diffusion from sites in the culture medium to the culture medium-slice interface, diffusion from culture medium into the slice over this interface and diffusion in the slice from the interface to metabolising sites. It is assumed that the three processes can be modelled simply as one process of exchange of compounds between culture medium and slice. Exchange is governed by Fick's law and parameterised with a diffusion coefficient, D_P. Consequently, exchange of drug is proportional to the difference between drug concentration in culture medium and slice: $D_P(C_{P,m} - C_{P,s})$, *'s'* for slice.

This transport model is refined by assuming that transport over the culture medium-slice interface is only possible for a fraction of the drug that is considered as "free", i.e., unbound to proteins or not sequestered in the lipid fraction of culture medium or slice. It will be assumed that binding and sequestration processes are unsaturated so that the unbound fraction is a constant, i.e., not depending on concentration levels. These free fractions are denoted by $f_{P,m}$ and $f_{P,s}$ for the parent compound in culture medium and slice, respectively. The exchange is assumed to be proportional to the difference between free concentration in culture medium and slice: $D_P(f_{P,m}C_{P,m} - f_{P,s}C_{P,s})$.

Once the drug has entered the liver slice, it can be metabolised after it has been bound to a metabolising enzyme. It is assumed that enzymatic binding is unsaturated, instantaneous and proportional to the drug free fraction. This way, the amount of drug metabolised is proportional to the free concentration: $K_s f_{P,s} C_{P,s}$. Here K_s denotes the metabolic clearance rate of the slice as a whole. On its turn, metabolite will diffuse and the amount of metabolite exchange between culture medium and slice is modelled as $D_M(f_{M,m}C_{M,m} - f_{M,s}C_{M,s})$, where *'M'* is for metabolite.

Note that in the slice model description 10 model parameters are involved: the initial concentration, $C_{P,m,0}$, of the parent compound, the culture medium and slice volumes V_m and V_s, which relate amounts of drug and metabolite to concentrations ($C = A/V$; A: amount), the diffusion coefficients for parent and metabolite D_P and D_M, the free fractions in culture medium and slice for parent and metabolite $f_{P,m}$, $f_{P,s}$, $f_{M,m}$ and $f_{M,s}$, and the parameter of interest, the slice metabolic rate constant K_s.

2.2 System model

The time course of the drug and metabolite concentration in culture medium and slice is governed by the mass balances for the amounts A of drug and metabolite in culture medium and slice. These mass balances define a system of differential equations (see Appendix A). In this system the physico-chemical model parameters appear in typical combinations, referred to as system parameters, which determine the kinetics of the compound and its metabolite. This

way, the physico-chemical slice model is reduced to a compartmental system (see figure 1, bottom left). This system consists of four compartments: parent compound in culture medium, parent compound in slice, metabolite in slice and metabolite in culture medium. The system parameters are the compound's initial amount, $A_{P,m,0} = V_m C_{P,m,0}$, and the following algebraic expressions in the model parameters:

$$d_{P,m} = \frac{f_{P,m} D_P}{V_m} \quad , \quad d_{P,s} = \frac{f_{P,s} D_P}{V_s} \quad , \quad k_s = \frac{f_{P,s} K_s}{V_s}$$
$$d_{M,m} = \frac{f_{M,m} D_M}{V_m} \quad , \quad d_{M,s} = \frac{f_{M,s} D_M}{V_s} \tag{1}$$

The solution of the system model will only contain the model parameters transformed as system parameters. As the system model completely describes the kinetics of the physico-chemical model, one can conclude that from any experimental set-up, one can at most identify a number of model parameters equal to the number of the system parameters. Because there are only 6 system parameters and 10 model parameters, *a priori* knowledge of some of the latter is required. It will be assumed throughout that the initial concentration of the parent compound in culture medium is known, leaving 5 system parameters and 9 model parameters.

2.3 Observable model

The analytical solution to the system equations can be found in Appendix A. It is expressed in terms of so called observable parameters, i.e., parameters that can be identified from sampling. E.g., when sampling the concentration of the parent compound in the slice, one observes an initial phase characterised by an increasing concentration, a terminal phase characterised by a decreasing concentration and the concentration levels themselves (see figure 1, bottom right). Initial phase rate and terminal phase rate are represented in the analytical solution by the two observable rate constants γ_1 and γ_2 of Appendix A. These rates appear to be algebraic expressions in the system parameters $d_{P,m}$, $d_{P,s}$ and k_s (see (2)). The third observable is a scaling factor that determines the concentration levels and which is expressed in terms of the initial and terminal phase rates and the system parameter $d_{P,m}$:

$$\gamma_1 = -\tfrac{1}{2}\left[d_{P,m} + d_{P,s} + k_s + \sqrt{\left(d_{P,m} + d_{P,s} + k_s\right)^2 - 4 d_{P,m} k_s} \right]$$
$$\gamma_2 = -\tfrac{1}{2}\left[d_{P,m} + d_{P,s} + k_s - \sqrt{\left(d_{P,m} + d_{P,s} + k_s\right)^2 - 4 d_{P,m} k_s} \right] \tag{2}$$
$$\sigma = \frac{d_{p,m}}{\gamma_1 - \gamma_2}$$

Notice that after fitting the observable parameters by fitting the model to the data, the system parameters can be calculated using (2) and from those the model parameters can be obtained, using (1).

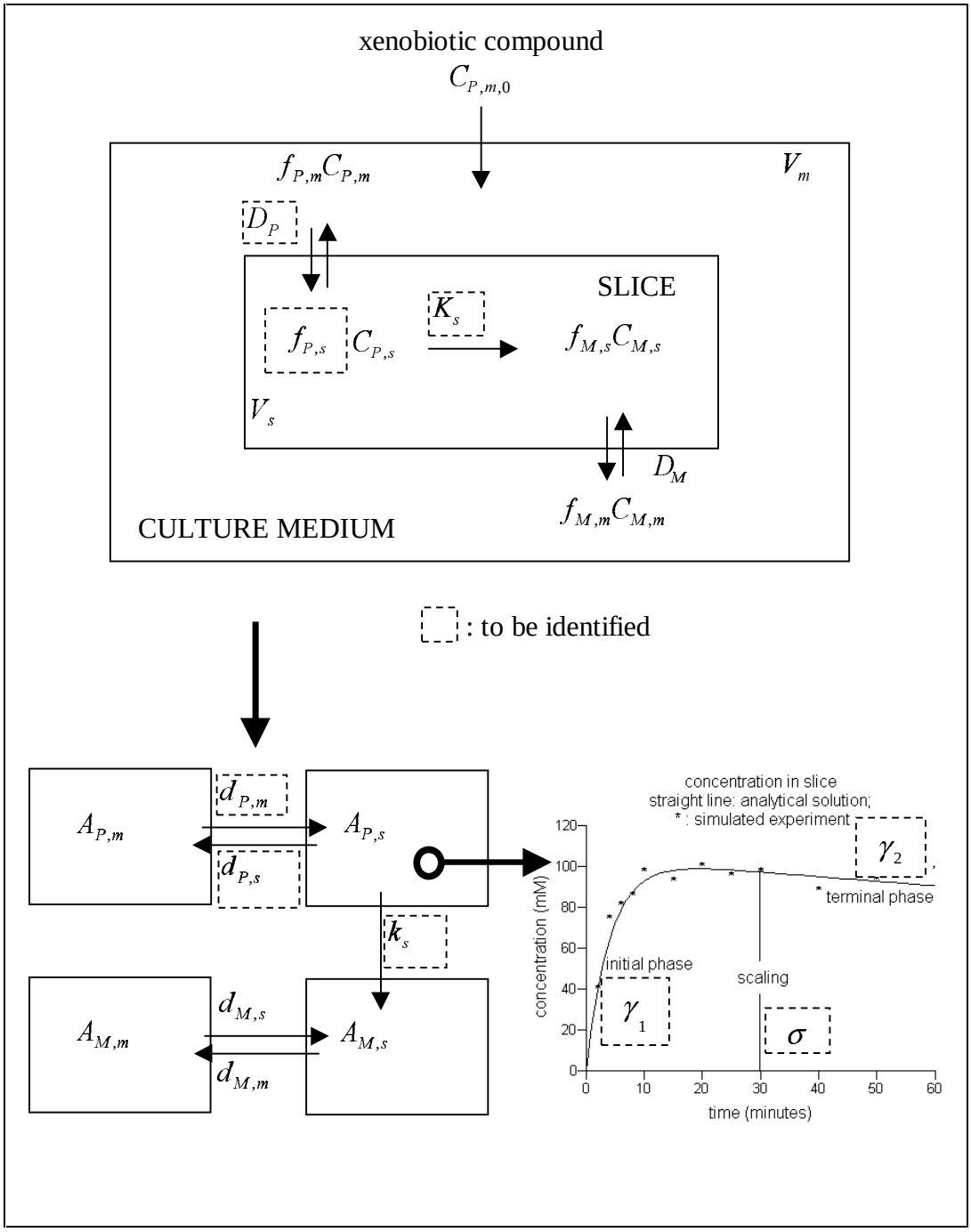

Figure 1. The physico-chemical slice model (top) and its model parameters; the system model (bottom left) together with its system parameters; the observable model for parent concentration in liver slice (bottom right) shows observations of parent compound in slice from a simulated experiment (*) from which the initial and terminal phase rates and concentration scaling can be identified, e.g., by fitting the analytical solution (straight line).

3. *In vitro - in vivo* extrapolation

This is a purposive research for the support of PBPK modelling in risk assessment. Therefore, the liver-slice experiment would be aimless if the results from this *in vitro* case could not be extrapolated to the *in vivo* case.

Extrapolating the clearance and free fraction obtained from the *in vitro* experiment, it will be assumed that the specific clearance of the liver, i.e. its metabolic rate per unit of volume, also referred to as specific intrinsic liver clearance, equals that of the slice: $K_l / V_l = K_s / V_s$. Also, it will be assumed that the unbound fractions in the liver equal those in the slice: $f_{P,l} = f_{P,s}$ and $f_{M,l} = f_{M,s}$. This is the mathematical expression of the assumption that the liver slice is an appropriate model for the liver as a whole.

In Appendix B, the mass balance for the liver compartment

$$\frac{dA_l}{dt} = Q_l(C_b - C_l / P_l) - f_{P,b} V_{l,t} \frac{K_s}{V_s} C_l / P_l \qquad (3)$$

where $P_l = v_{l,v} + f_{P,b} / f_{P,s} \cdot v_{l,t}$, is derived in terms of the slice model parameters $f_{P,s}, K_s,$ and V_s. Here the liver-blood partition coefficient is, Q is blood flow, *'l'* denotes liver and *'b'* blood, *'v'* denotes venous subcompartment in the liver and *'t'* the tissue subcompartment.

From this formulation it can be concluded that

1. For the sake of *in vivo* PBPK modelling the specific intrinsic clearance K_s / V_s should be identified. However, this specific clearance can only be identified when the slice's free fraction is known (see (1)), which will generally not be the case. The free fraction could be identified when the diffusion parameter D_P and the slice volume V_s were known (see (1)). The diffusion parameter, which will almost certainly not be known, can only be identified when the culture medium's free fraction and volume are known (see (1)). So, it will be assumed that these last 2 parameters are known together witn the slice's volume: only then the intrinsic specific clearance and the free fraction of parent compound in slice can be identified, see also chapter 4
2. For PBPK modelling the blood-liver partition coefficient should be known, which implies additional identification of the free fraction of the compound in slice or a separate experimental determination of the liver-blood partition coefficient P_l. The latter determination is difficult, because the liver is a metabolising organ: a constant ratio between concentrations in perfusing blood and liver should be corrected for to obtain the true partition coefficient (see e.g. Chen and Gross (1979)).
3. For PBPK modelling the free fraction of the compound in blood, $f_{P,b}$ should be known.

4. The formal identification analysis problem

4.1 Analytical solution analysis

From the analysis in chapter 3 it follows that identification of the specific intrinsic liver clearance K_s/V_s is required. The way this model parameter is expressed in the system parameters is in the parameter $k_s = f_{P,s}K_s/V_s$. So, if this system parameter is not identifiable from an experiment, then the specific intrinsic clearance itself cannot be identified. From the appendix, expression (A6), it follows that

$$k_s = \frac{\gamma_1\gamma_2}{\sigma(\gamma_1 - \gamma_2)} \tag{4}$$

where γ_1 is the initial rate of change of parent concentration in slice, γ_2 is the terminal rate of change of parent concentration in slice, and σ is the actual concentration scaling factor (see (A5)). So, if one of these observable parameters is not identifiable from an experiment, then k_s nor K_s/V_s will be identifiable.

The other way around, when these three observable parameters are identifiable, then not only the system parameter k_s can be identified, but also the other two parameters $d_{P,s}$ and $d_{P,m}$ (from (A6)):

$$\begin{aligned} d_{P,m} &= \sigma(\gamma_1 - \gamma_2) \\ d_{P,s} &= -\gamma_1 - \gamma_2 - d_{P,m} - k_s \end{aligned} \tag{5}$$

It will be assumed that in addition to the specific intrinsic clearance, the compound's free fraction in slice and the diffusion parameter are unknown. Then, from (1), (4) and (5) it is easily seen that the three model parameters K_s, $f_{P,s}$ and D_P are identifiable once the three observable parameters are identifiable from an experiment or, equivalently, once the three system parameters are identifiable.

However, from these same equations it follows that identification requires knowledge of the model parameters V_m, $f_{P,m}$ and V_s. This requirement is easily met for the first of these three, for the second it requires judicious choice of a culture medium or an independent experiment for its identification, while the last requires careful determination of the slice volume and an examination of the slice histology during experimental conditions.

Throughout, it will be assumed that the model parameters A_0, $f_{P,m}$, V_m and V_s are known. Then, from (A5) and (A8) it can be inferred that when the parent compound is administrated in medium only, identifiability is formally guaranteed when sampling the parent compound in slice or in medium. However, in this case identifiability is impossible when sampling the metabolite pooled from medium and slice. This can be inferred from (A7) which lacks the scaling factor as third observable parameter. Because the amount of parent compound and metabolite is complementary, one can immediately conclude that sampling the pooled parent compound is insufficient too.

For completeness, the physico-chemical model was introduced together with transport and binding of metabolite. One may wonder, if non-identifiability when sampling pooled metabolite can be regained by sampling metabolite in slice and medium separately. However, (A1) indicates that this introduces at least 2 extra model parameters that most likely are not known: the diffusion parameter for metabolite and the metabolite's free fraction in slice. Thus, sampling metabolite in medium and slice separately will not contribute to identification of intrinsic clearance and free fraction.

Formally it is sufficient to consider a 2-compartment system model, i.e., parent compound in medium and parent compound in slice, instead of the 4-compartment system model resulting from the complete physico-chemical model. For reasons discussed below, however, the 3-compartment system model - parent compound in medium and parent compound in slice and metabolite pooled from medium and slice - will be considered in the next chapters.

4.2 Compartmental system analysis

In section 4.1 the question of identifying model parameters has been answered by considering the analytical solution. Other identification methods are extensively described in the literature on compartmental analysis (Anderson, 1983; Carson *et al*. 1983; Godfrey, 1983; Jacquez, 1996; van den Hof, 1996). One of these methods considers the system model in Laplace transformed time instead of in real time. By this Laplace transformation the system of differential equations is transformed in a system of linear equations. The coefficient matrix of this last system consists of elements which are linear expressions in the system parameters and the transformed time co-ordinate. Depending on the experimental set-up, i.e. which (combinations of) compartments are sampled and which compartments are "forced", i.e. the parent compound is administrated to, a few elements of the inverse matrix of this, so called, transfer matrix have to be calculated. So, a set of non-linear equations in the system model parameters is obtained, the solution of which determines their identifiability. See Appendix C for details.

Of course, this method yields results equivalent to using the analytical solution. Nevertheless, the method is applied to analyse the slice experiment next to the use of the analytical solution. The results of the analysis can be found in section Table 1. The method is also applied to analyse the slice experiment under the rather academic condition that initially the parent compound is in the slice instead of in culture medium. This last example, academic as it may be, will show the importance of designing an experiment by taking into account identifiability aspects. Results are presented in Table 2.

The tables 1 and 2 show the formal minimal requirements with respect to sampling in order to identify the intrinsic clearance, the compounds free fraction in the slice and the diffusion parameter. It appears that when the parent compound is administered to culture medium, sampling the parent compound in culture medium or parent compound in slice leads to identification. All other sampling strategies, i.e., strategies which do not include the separate sampling of the parent compound in culture medium or the parent compound in slice are insufficient. In contrast, when the parent compound is administrated to slice, sampling pooled parent compound or pooled metabolite, identification is formally guaranteed. Also, sampling parent compound in slice will lead to identification as in the case above. However, sampling parent compound in culture medium will lead to 2 interchangeable solutions for the system parameters $d_{P,m}$ and k_s and consequently to 2 different sets of model parameters.

*Table 1. Identifiability table of an in vitro liver-slice experiment when **administration** of the parent compound is **in culture medium**. The first column shows the sampling site(s) and the second whether identifiability is possible or not.*

Sampling parent compound in:		
culture medium		YES
liver slice		YES
culture medium and slice; separate		YES
culture medium and slice; pooled		no
Sampling metabolite in:		
culture medium		no
liver slice		no
culture medium and liver slice; separate		no
culture medium and liver slice; pooled		no
Sampling parent compound and metabolite		
parent compound in:	metabolite in:	
culture medium	culture medium	YES
	liver slice	YES
	culture medium and liver slice; separate	YES
	culture medium and liver slice; pooled	no
liver slice	culture medium	YES
	liver slice	YES
	culture medium and liver slice; separate	YES
	culture medium and liver slice; pooled	no
culture medium and liver slice; separate	culture medium	YES
	liver slice	YES
	culture medium and liver slice; separate	YES
	culture medium and liver slice; pooled	no
culture medium and liver slice; pooled	culture medium	no
	liver slice	no
	culture medium and liver slice; separate	no
	culture medium and liver slice; pooled	no

*Table 2. Identifiability table of an in vitro liver-slice experiment when **administration** of the parent compound is **in liver slice**. The first column shows the sampling site(s) and the second whether identifiability is possible or not.*

Sampling parent compound in:		
culture medium		no[1]
liver slice		YES
culture medium and slice; separate		YES
culture medium and slice; pooled		YES
Sampling metabolite in:		
culture medium		no[1]
liver slice		no[1]
culture medium and liver slice; separate		YES
culture medium and liver slice; pooled		YES
Sampling parent compound and metabolite		
parent compound in:	metabolite in:	
culture medium	culture medium	YES
	liver slice	YES
	culture medium and liver slice; separate	YES
	culture medium and liver slice; pooled	YES
liver slice	culture medium	YES
	liver slice	YES
	culture medium and liver slice; separate	YES
	culture medium and liver slice; pooled	YES
culture medium and liver slice; separate	culture medium	YES
	liver slice	YES
	culture medium and liver slice; separate	YES
	culture medium and liver slice; pooled	YES
culture medium and liver slice; pooled	culture medium	YES
	liver slice	YES
	culture medium and liver slice; separate	YES
	culture medium and liver slice; pooled	YES

[1] 2 or 3 different solutions to the identification problem are possible: additional information may help to choose the right one.

5. The identification problem in practice

5.1 Simulation of real-world experiments

In chapter 4 conditions were derived for experimental set-up, such that the experiment would lead to the identification of the desired model parameters. These conditions were both necessary and formally sufficient. However, formal sufficiency does not imply sufficiency in practice: only a limited number of samplings can be carried out, e.g., because of the duration of liver slice viability, lab capacity, and so on. Also, the observed samples contain errors, e.g. due to the fact that every sample originates from a different (group of) slice(s).

Slob *et al.* (1997) discuss some practical considerations in their analysis of the identification problem. They present a rule of thumb for the minimum number of data points required for identification. For the liver slice experiment that number, N, is given by $N = 2n - r + 1 + m(n - r + 1) = 9$, where $n = 3$ is the number of state variables (parent compound in culture medium, parent compound in slice and pooled metabolite), $m = 1$ is the number of inputs (non-zero initial concentration in culture medium) and $r = 1$ is the number of observed compartments (parent compound in slice, e.g.). This result is obtained under the condition that measurements are exact and that there are no no limitations with respect to the duration of the experiment.

In order to investigate sufficiency in practice, when no exact measurements are available, data of the outcome of a typical slice experiment with a duration of only 1 hour have been simulated. Five sets of data have been simulated by random perturbation of theoretical values at certain points in time, to be considered as the times of observation (2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50 and 60 minutes). These data served the back-estimation of the model parameters, by fitting the model to them.

5.2 Identification by fitting to simulated data

Since the relation between observable and system parameters and between system and model parameters is known, one has the luxury to choose whether to fit the observable parameters, the system parameters or the model parameters itself. Notice that the system analysis showed that no more than three parameters can be identified. In a classical kinetic approach, one would easily decide that, because two compartments are involved, four parameters should be fitted: a rate parameter and a coefficient for each of the two corresponding exponentials. In a modelling approach, one would easily overlook the fact that the combination of parameters admits fitting only three and, e.g., the free fraction in culture medium should be known lest the experiment have any sense.

It can be shown that even if the fitted observable parameters are within their physical range, the physically acceptable range for one of the system parameters can be violated: the estimated values of the initial and terminal rate do not guarantee a resulting positive value for $d_{P,s}$. A negative value for $d_{P,s}$ leads to negative values for both K_s and $f_{P,s}$. This has been verified from fitting the observable parameters to simulated data. Likewise, even if the fitted system parameters are within their acceptable range, the physically acceptable range for the model parameters can be violated: the value for the free fraction derived from the fitted system parameters can be greater than 1. This has been verified from fitting the system parameters to

simulated data. To prevent these problems we will only report the fitting of the model parameters.

The three parameters, diffusion of the parent compound D_P , its free fraction in liver tissue $f_{P,s}$ and the liver slice clearance K_s, have been fitted against the simulated data. Model fits were performed on each of the 5 sets of simulated data separately. The nominal values for the model parameters to be fitted were chosen to be

$$K_s = 0.009\,[\text{mL/min}],\ f_{P,s} = 0.3,\ D_P = 0.003\,[\text{mL/min}]$$

The other model parameters are

$$f_{P,m} = 0.3, V_m = 1\,[\text{ml}], V_s = 0.015\,[\text{ml}]$$

This choice of parameters results in a concentration-time curve that attains its maximum at about $t = 25$ minutes and then slightly decreases. In variation to this set of parameters, the cases $K_s = 0.0009$ and $K_s = 0.09$ have been considered too. Notice that from the identifiability analysis it follows that there is no sense in modelling the metabolite concentrations in slice and culture medium separately and consequently its free fraction and diffusion coefficient value do not matter.

We started fitting parameters for the case that the liver slice clearance $K_s = 0.0009$. It appeared that no reliable estimate of K_s nor of $f_{P,s}$ could be obtained: for the five different sets of simulated observations, estimates for K_s ranged from 7.4×10^{-7} to 2.3, while corresponding values for $f_{P,s}$ ranged from 0.39 to 0.00048. Fitted values depended on the initial estimate of parameters. The fitted parameter values for clearance and free fraction were strongly correlated. The fitted value for the diffusion parameter, which is not of interest, was always within 6% of the true value. It was guessed that due to the short duration of the experiment (1 hour), compared to the half-life time of the terminal phase (56 hours) practically no "information" of the terminal phase is present in the slice concentration time course: when the concentration in the slice levels off its maximum value to its value at 1 hour, the concentration difference is in the same order as the simulated experimental errors. It was hoped that the simulated samples of pooled metabolite would contain this information, because the corresponding concentration range is much greater than the simulated errors.

Parameters were fitted on both concentration time courses simultaneously. This appeared to be a successful strategy: K_s and $f_{P,s}$ were fitted within error bounds of 12% and 6% respectively. Thus, while sampling metabolite concentration data only do not fullfill the necessary condition for identification and while sampling only slice concentration data appear to be insufficient for identification, the combined data fulfil both the necessary and sufficient condition.

However, applying this heuristic procedure to the case $K_s = 0.009$, i.e. fitting parameters on both concentration time courses simultaneously, it appeared that values found for K_s and $f_{P,s}$ could deviate a factor 2.4 and 0.4 respectively from their true values. And even worse results were obtained for the case $K_s = 0.09$, where deviating factors were 205 and 0.005 respectively. So, an analysis with more mathematical rigour seemed to be needed. This analyses is discussed in the next three sections.

5.3 Model sensitivity with respect to the observable parameters

Firstly, the sensitivity of the time course of parent compound concentration in slice and culture medium and the sensitivity of the time course of pooled metabolite concentration to the observable parameters is analysed in Appendix D. The rationale for this analysis is that when data are sampled only during a time span when the sensitivity to one of the observable parameters is nil or very low, fitting the model parameters may result in a value for that parameter that deviates substantially from the true one. In figure 2 the result of this analysis is depicted.

From this figure it appears that when sampling only the parent concentration in slice, sample time points should extend to times in the order of the half-life time for the terminal phase, i.e. about 56, 17 and 13 **hours** respectively for $K_s = 0.0009$, 0.009 and 0.09 [ml/min]. Only then, a reasonable estimate of the terminal rate can be obtained. Surprisingly, when fitting parameters simultaneously to data sampled from parent compound in slice and pooled metabolite, there is no need to let the experiment last longer than about 3 times the half-life of the initial rate, i.e. about 25, 9 and 1.2 **minutes** respectively for $K_s = 0.0009$, 0.009 and 0.09 [ml/min]: during that period only, the concentration time course in slice is sensitive for the initial rate. During that period, also the sensitivity of the concentration time course of pooled metabolite to the terminal phase is strong.

5.4 Model parameter sensitivity with respect to the observable parameters

Secondly, in Appendix E it is analysed that the sensitivity of the model parameters with respect to variation in the observable parameters can be substantial. For instance, multiplicative errors in the value for the terminal phase rate are blown up, due to dividing those errors by a term that can approximate zero closely. As the product $f_{P,s}K_s$ of fitted parameters will not be blown up, consequently the fitted value for $f_{P,s}$ will be very small. So, fitting the model parameters can result in values for K_s and $f_{P,s}$ with a very large deviation from their true values, while the corresponding terminal rate parameter deviates far less from its true value. As also multiplicative errors of the initial phase rate and the scaling factor are involved in the blow-up factor, these errors will contribute too.

Simulations were run, simulating an experiment lasting 60 minutes for $K_s = 0.0009$, 0.009 and 0.09 [ml/min] respectively. Fitting the model parameters on data sampled from parent compound in slice only, the observable parameters obtained from them deviated from the true ones as follows. The initial rate deviated by factors ranging 0.93-1.12, 0.95-1.19 and 0.73-3.84 for $K_s = 0.0009$, 0.009 and 0.09 [ml/min] respectively, while the terminal rate deviated factors ranging ≈ 0-4.63, 0.27-1.43 and 0.74-4.05. The range of factors for the estimated parameters was much greater. The clearance deviated by factors ranging ≈ 0-2700, 0.08-436 and 6.9-13, while the free fraction deviated factors ranging ≈ 0-1.31, ≈ 0-3.33 and 0.07-0.56.

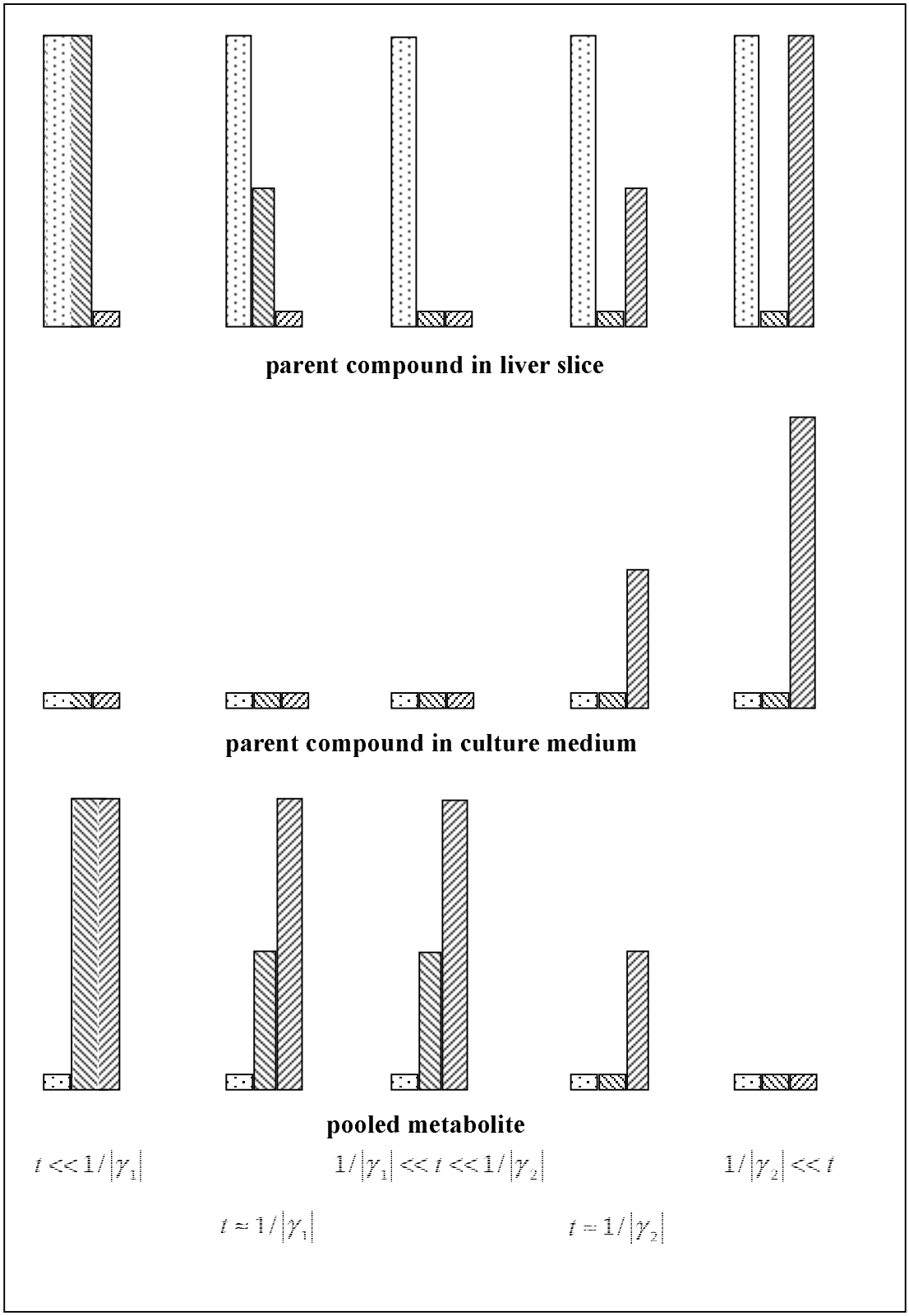

Figure 2. Sensitivity of the concentration of the parent compound in liver slice (top) and culture medium (middle) and pooled metabolite (bottom) for variations in the model parameters σ (dotted pattern), γ_1 (line downward diagonal) and γ_2 (line upward diagonal) during several time trajectories. The smallest bars denote minor or no sensitivity, the largest bars greatest sensitivity.

5.5 Error propagation from observable to model parameters

Thirdly, in Appendix E an error analysis shows that small relative errors in the observable parameters can be blown up to an error in K_s: these errors are multiplied by the factor K_s / D_P and can cause a denominator in the expression of the error for K_s to approximate zero closely. The other way around, this implies that substantial errors in the model parameter estimations for clearance and free fraction are attenuated to only small ones in the observable parameters. See figure 3.

Simulations were run, simulating an experiment lasting about 25, 9 and 1.2 minutes for $K_s = 0.0009$, 0.009 and 0.09 [ml/min] respectively. Fitting the model parameters on data sampled from parent compound in slice and pooled metabolite simultaneously, the observable parameters obtained from them deviated from the true ones with relative errors in the same order as when fitting to the same amount of data sampled during a period of 60 minutes. This confirms the observation above that it is sufficient to have samples obtained during the period that the sensitivity of the solution in slice for the initial phase is still substantial. Notwithstanding the small relative errors in the observable parameters derived from the fitted model parameters, the latter appeared to be more and much more in error for $K_s = 0.009$ and 0.09 respectively.

E.g., for one of the five sets of simulated data with $K_s = 0.09$ the fit of model parameters results in values for the observable ones with relative errors of 3.3, 1.3 and 1.2% for the initial rate, terminal rate and scaling factor respectively. However, the values for K_s and $f_{P,s}$ deviate with factors 204 and 4.9×10^{-3} respectively (note their product to be almost 1). In this sense, the identification problem is ill defined whenever the slice clearance value exceeds the value of the model diffusion coefficient. Notice that this last value depends on the experimental set-up, such as slice thickness and culture medium volume.

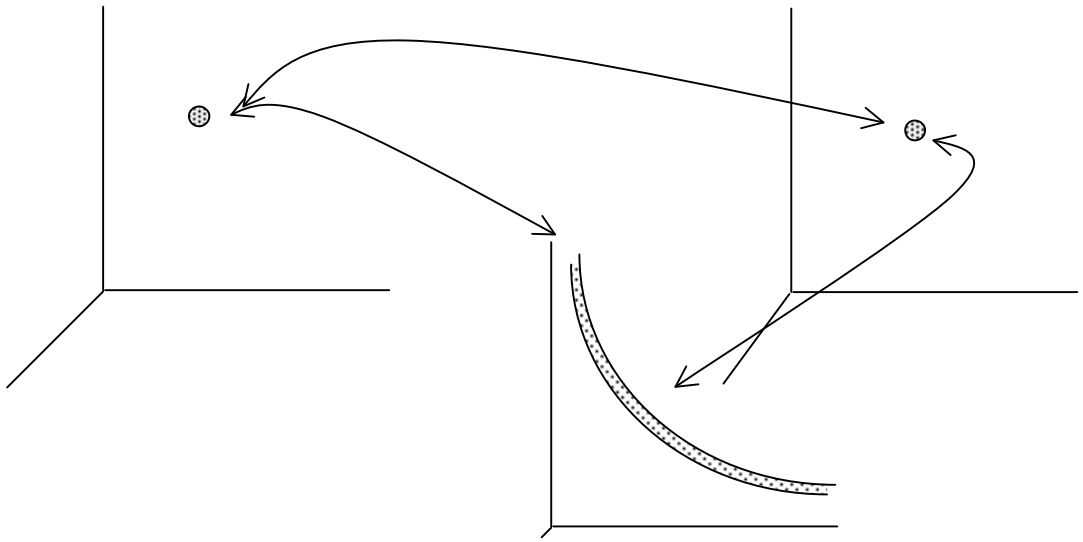

Figure 3. Showing "blow up" of range of model parameters with respect to range of observable and system parameters.

6. Conclusions

Performing *in vitro* liver slice experiments for the identification of the metabolism rate of compounds appears to be a delicate task. Even in a best case analysis, i.e., linear binding and metabolism of the parent compound and transport described by only one diffusion parameter, identification appears to be possible only if several requirements are met. Also, one of the pitfalls of the identification analysis itself turned out to be the ignorance of practical aspects and to rely on a formal analysis only. While the formal analysis prescribes sampling the parent compound in slice or culture medium, the practical analysis sharpens this to sampling the parent compound in slice. Moreover, the practical analysis prescribes the additional sampling of pooled metabolite. From a pilot study one should obtain some information as to the sample time scheme. Even if all these requirements are met, analysis shows that identification results can be unreliable, whenever the slice intrinsic clearance exceeds the diffusion coefficient value, the more so, when the clearance is much greater than the diffusion coefficient.. This condition can only be verified afterwards.

One way to overcome this and enlarge the diffusion coefficient value is using hepatocytes instead of slices. Compared to a slice consisting of ten viable cell layers, the contact surface between cells and culture medium will be about tenfold for the same number of hepatocytes. Also, intra-slice diffusion will slow down the experimentally effective diffusion coefficient value and it may be expected that using hepatocytes enlarges this value by a factor 10 to 100, say 30. The slice model can be applied modelling experiments with hepatocytes as well: the only difference being the diffusion rate between culture medium and slice or hepatocytes. However, when a compound is metabolised very fast, even such an experiment with hepatocytes instead of slices would not help any longer.

The identification problem appears to be a tough one, because of the strong correlation between the intrinsic clearance and the compound free fraction in liver. Experimental data would be more meaningful and clearance could be identified with much greater reliability when the free fraction in liver has been identified from another, independent experiment. Undoubtedly, such an experiment would need a mathematical model for the interpretation of the experimental data...

Extrapolation of the *in vitro* parameters to *in vivo* seems to be straightforward, when both the *in vitro* and *in vivo* models are expressed in their basic kinetic processes. The compound's free fraction in blood is required for extrapolation, which seems to be analogous to the requirement that the compound's free fraction in culture medium should be known *a priori* for the sake of identification of the required model parameters.

In short:

- for the sake of identification the compound's free fraction in culture medium, the culture medium vollume and the liver-slice volume must be known. Identification of the compound's free fraction in liver slice is highly desirable, if not requisite, when identifying intrinsic clearance for a fast metabolising compound.
- for the sake of identification, given the experimental limitations, sampling the paren compound in slice and pooled metabolite is required
- when the value of the intrinsic clearance, identified from the data, exceeds the value of the diffusion rate more than, say, a factor of three, identification results for clearance and compound's free fraction in slice are unreliable and, e.g., one has to resort to hepatocytes or the independent identification of the compound's free fraction in slice
- for the sake of extapolation the compound's free fraction in blood must be known

Acknowledgement

Based on his nice analyses of samples of metabolites of tolbutamide from a preliminary experiment Martin Hamzink emphasised that experimental results on metabolites should be used in solving the identification problem. Theoretically he was wrong, as a man of practice he was right. Being a man of practice, Klaas van Twillert suggested the rather academic "what if" administrating parent compound to slice instead of to culture medium.

References

D.H. Anderson; *Compartmental modeling and tracer kinetics*; Springer-Verlag, Berlin etc., 1983.

Carson, E.R., Cobelli, C., and L. Finkelstein; *The mathematical modeling of metabolic and endocrine systems*; John Wiley & Sons, New York etc. 1983.

K. Godfrey; *Compartmental models and their application*; Academic Press, London etc., 1983.

van den Hof, J. M.; *System theory and system identification of compartmental systems*, Ph.D. Thesis, RUG, Groningen, 1996

J.A. Jacquez; *Compartmental analysis in biology and medicine* (third ed.); BioMedware, Ann Arbor, 1996.

Slob, W., Janssen, P.H.M., and J.M. van den Hof; Structural identifiability of PBPK models: practical consequences for modeling strategies and study designs; *Critical Rev. in Toxicol.*; **27** (1997) 261-272

Worboys, P.D., Bradbury A., and J.B. Houston; Kinetics of drug metabolism in rat liver slices. Rates of oxidation of ethoxycoumarin and tolbutamide, examples of high- and low-clearance compounds; *Drug Metabolism and Disposition*; **23** (1995) 393-397

Worboys, P.D., Bradbury A., and J.B. Houston; Kinetics of drug metabolism in rat liver slices. II. Comparison of clearance by liver slices and freshly isolated hepatocytes; *Drug Metabolism and Disposition*; **24** (1996) 676-681

Worboys, P.D., Bradbury A., and J.B. Houston.; Kinetics of drug metabolism in rat liver slices. III. Relationship between metabolic clearance and slice uptake rate; *Drug Metabolism and Disposition*; **25** (1997) 460-467

Appendix A Model solution

Let the amounts in culture medium and slice of parent compound and metabolite, respectively, be given by $A_{P,m}$, $A_{P,s}$, $A_{M,m}$ and $A_{M,s}$. The rate of change of these quantities is given by the following set of differential equations:

$$\begin{aligned}
\dot{A}_{P,m} &= -D_P(f_{P,m}C_{P,m} - f_{P,s}C_{P,s}) \\
\dot{A}_{P,s} &= D_P(f_{P,m}C_{P,m} - f_{P,s}C_{P,s}) - K_s f_{P,s}C_{P,s} \\
\dot{A}_{M,s} &= K_s f_{P,s}C_{P,s} - D_M(f_{M,s}C_{M,s} - f_{M,m}C_{M,m}) \\
\dot{A}_{M,m} &= D_M(f_{M,s}C_{M,s} - f_{M,m}C_{M,m})
\end{aligned} \tag{A1}$$

Where $C_{P,m} = A_{P,m}/V_m$, $C_{P,s} = A_{P,s}/V_s$, $C_{M,s} = A_{M,s}/V_s$ and $C_{M,m} = A_{M,m}/V_m$ are the concentrations of parent compound and metabolite respectively, $f_{P,m}$ and $f_{P,s}$ are the unbound fractions of parent compound and $f_{M,s}$ and $f_{M,m}$ unbound fractions of the metabolite, K_s is the intrinsic metabolic clearance rate and D_P and D_M are diffusion parameters.
As initial conditions we take $A_{P,m}(0) = A_0$, $A_{P,s}(0) = 0$, $A_{M,s}(0) = 0$ and $A_{M,m}(0) = 0$.

First, the following system parameters, which are algebraic expressions in the model parameters, are introduced:

$$\begin{aligned}
d_{P,m} &= \frac{f_{P,m}D_P}{V_m}, \quad d_{P,s} = \frac{f_{P,s}D_P}{V_s}, \quad k_s = \frac{f_{P,s}K_s}{V_s} \\
d_{M,m} &= \frac{f_{M,m}D_M}{V_m}, \quad d_{M,s} = \frac{f_{M,s}D_M}{V_s}
\end{aligned} \tag{A2}$$

Consider the sum $A_M = A_{M,m} + A_{M,s}$ of metabolite in the slice-culture medium pool and let the amount of parent compound in culture medium be complementary: $A_{P,m} = A_0 - A_{P,s} - A_M$. Then (A1) reduces to:

$$\begin{aligned}
\dot{A}_{P,s} &= d_{P,m}\left(A_0 - A_{P,s} - A_M\right) - d_{P,s}A_{P,s} - k_s A_{P,s} \quad & A_{P,s}(0) = 0 \\
\dot{A}_M &= k_s A_{P,s} & A_M(0) = 0
\end{aligned} \tag{A3}$$

Taking the second time derivative in the first line of (A3), and substituting the second line, the resulting second order differential equation for $A_{P,s}$

$$\begin{cases}
\ddot{A}_{P,s} = -\left(d_{P,m} + d_{P,s} + k_s\right)\cdot \dot{A}_{P,s} - d_{P,m}k_s A_{P,s} \\
A_{P,s}(0) = 0 \quad \dot{A}_{P,s}(0) = d_{P,m}A_0
\end{cases} \tag{A4}$$

has as solution

$$A_{P,s}(t) = \sigma A_0 \left(\exp(\gamma_1 t) - \exp(\gamma_2 t)\right) \tag{A5}$$

where

$$\begin{aligned}
\gamma_1 &= -\tfrac{1}{2}\left[d_{P,m} + d_{P,s} + k_s + \sqrt{\left(d_{P,m} + d_{P,s} + k_s\right)^2 - 4 d_{P,m} k_s} \right] \\
\gamma_2 &= -\tfrac{1}{2}\left[d_{P,m} + d_{P,s} + k_s - \sqrt{\left(d_{P,m} + d_{P,s} + k_s\right)^2 - 4 d_{P,m} k_s} \right] \\
\sigma &= \frac{d_{P,m}}{\gamma_1 - \gamma_2}
\end{aligned} \tag{A6}$$

Consequently, the corresponding solution for the sum of amounts of metabolite in slice and culture medium is

$$A_M(t) = \int_0^t k_s A_{P,s}(\tau)\, d\tau = \left[1 - \frac{\gamma_1 \exp(\gamma_2 t) - \gamma_2 \exp(\gamma_1 t)}{\gamma_1 - \gamma_2} \right] \cdot A_0 \tag{A7}$$

and the solution for the amount of parent compound in culture medium is :

$$A_{P,m}(t) = \sigma A_0 \left[(1 + \frac{\gamma_1}{d_{P,m}}) \exp(\gamma_2 t) - (1 + \frac{\gamma_2}{d_{P,m}}) \exp(\gamma_1 t) \right] \tag{A8}$$

The problem for the amounts of metabolite in culture medium and slice separately can now be obtained from (A1) and (A5):

$$\begin{aligned}
\begin{pmatrix} \dot{A}_{M,s} \\ \dot{A}_{M,m} \end{pmatrix} &= \underbrace{\begin{pmatrix} -d_{M,s} & d_{M,m} \\ d_{M,s} & -d_{M,m} \end{pmatrix}}_{=\underline{\underline{M}}} \begin{pmatrix} A_{M,s} \\ A_{M,m} \end{pmatrix} + \begin{pmatrix} \dot{A}_M \\ 0 \end{pmatrix} \\
&= \begin{pmatrix} -d_{M,s} & d_{M,m} \\ d_{M,s} & -d_{M,m} \end{pmatrix} \begin{pmatrix} A_{M,s} \\ A_{M,m} \end{pmatrix} + \begin{pmatrix} k_s A_{P,s} \\ 0 \end{pmatrix} \qquad \begin{pmatrix} A_{M,s}(0) \\ A_{M,m}(0) \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \end{pmatrix}
\end{aligned} \tag{A9}$$

The formal solution to this problem is

$$\begin{pmatrix} A_{M,s}(t) \\ A_{M,m}(t) \end{pmatrix} = \exp\left(t\underline{\underline{M}}\right) \int_0^t \exp\left(-\tau \underline{\underline{M}}\right) \begin{pmatrix} k_s A_{P,s}(\tau) \\ 0 \end{pmatrix} d\tau \tag{A10}$$

The explicit solution can be found from the observation that

$$\underline{\underline{M}} \begin{pmatrix} 1 \\ -1 \end{pmatrix} = -\left(d_{M,m} + d_{M,s}\right) \cdot \begin{pmatrix} 1 \\ -1 \end{pmatrix} \qquad \underline{\underline{M}} \begin{pmatrix} d_{M,m} \\ d_{M,s} \end{pmatrix} = 0 \cdot \begin{pmatrix} d_{M,m} \\ d_{M,s} \end{pmatrix} \tag{A11}$$

So, 0 and $\gamma_M = -\left(d_{M,m} + d_{M,s}\right)$ are the two eigenvalues of $\underline{\underline{M}}$.
Thus, the matrix consisting of the two eigenvectors is

$$\underline{\underline{S}} = \begin{pmatrix} d_{M,m} & 1 \\ d_{M,s} & -1 \end{pmatrix} \tag{A12}$$

and we have

$$\underline{\underline{M}}\,\underline{\underline{S}} = \underline{\underline{S}} \underbrace{\begin{pmatrix} 0 & 0 \\ 0 & \gamma_M \end{pmatrix}}_{\underline{\underline{\Lambda}}} \tag{A13}$$

and

$$\exp\left(t\underline{\underline{M}}\right) = \underline{\underline{S}} \underbrace{\begin{pmatrix} 1 & 0 \\ 0 & e^{\gamma_M t} \end{pmatrix}}_{\exp(t\underline{\underline{\Lambda}})} \underline{\underline{S}}^{-1} \tag{A14}$$

The inverse of $\underline{\underline{S}}$ is

$$\underline{\underline{S}}^{-1} = \frac{-1}{\gamma_M} \begin{pmatrix} 1 & 1 \\ d_{M,s} & -d_{M,m} \end{pmatrix} \tag{A15}$$

Substituting these expressions in (A10) one obtains

$$\begin{aligned} \begin{pmatrix} A_{M,s}(t) \\ A_{M,m}(t) \end{pmatrix} &= \underline{\underline{S}} \exp(t\underline{\underline{\Lambda}}) \underline{\underline{S}}^{-1} \int_0^t \underline{\underline{S}} \exp(-\tau\underline{\underline{\Lambda}}) \underline{\underline{S}}^{-1} \begin{pmatrix} k_s A_{P,s}(\tau) \\ 0 \end{pmatrix} d\tau \\ &= \frac{-k_s}{\gamma_M} \underline{\underline{S}} \exp(t\underline{\underline{\Lambda}}) \int_0^t A_{P,s}(\tau) \begin{pmatrix} 1 & 0 \\ 0 & e^{-\gamma_M \tau} \end{pmatrix} \begin{pmatrix} 1 \\ d_{M,s} \end{pmatrix} d\tau \\ &= \frac{-\sigma\, k_s A_0}{\gamma_M} \underline{\underline{S}} \begin{pmatrix} 1 & 0 \\ 0 & e^{\gamma_M t} \end{pmatrix} \times \\ &\qquad \begin{pmatrix} \gamma_1^{-1}\left(\exp(\gamma_1 t) - 1\right) - \gamma_2^{-1}\left(\exp(\gamma_2 t) - 1\right) \\ \left[d_{M,s}\left(-\gamma_M + \gamma_1\right)^{-1}\left(\exp\left(\left(-\gamma_M + \gamma_1\right)t\right) - 1\right) - \right. \\ \left. \left(-\gamma_M + \gamma_2\right)^{-1}\left(\exp\left(\left(-\gamma_M + \gamma_2\right)t\right) - 1\right) \right] \end{pmatrix} \end{aligned} \tag{A16}$$

From this expression it follows that

$$\begin{aligned} A_{M,s}(t) = \frac{-k_s \sigma A_0}{\gamma_M} \Bigg(& \frac{d_{M,m}}{\gamma_1}\left(e^{\gamma_1 t} - 1\right) + \frac{d_{M,s}}{\gamma_1 - \gamma_2}\left(e^{\gamma_1 t} - e^{\gamma_M t}\right) \\ & - \frac{d_{M,m}}{\gamma_2}\left(e^{\gamma_2 t} - 1\right) - \frac{d_{M,s}}{\gamma_2 - \gamma_M}\left(e^{\gamma_2 t} - e^{\gamma_M t}\right) \Bigg) \end{aligned} \tag{A17}$$

with $\lim_{t\to\infty} A_{M,s}(t) = \frac{-\sigma k_s A_0}{\gamma_M} d_{M,m}\left(\frac{1}{\gamma_1} - \frac{1}{\gamma_2}\right) = \frac{-\sigma k_s A_0}{\gamma_M} d_{M,m} \frac{-(\gamma_1 - \gamma_2)}{d_{P,m} k_s} = \frac{d_{M,m} A_0}{d_{M,m} + d_{M,s}}$

and

$$A_{M,m}(t) = \frac{-\sigma k_s A_0}{\gamma_M}\left(\frac{d_{M,s}}{\gamma_1}\left(e^{\gamma_1 t} - 1\right) - \frac{d_{M,s}}{\gamma_1 - \gamma_M}\left(e^{\gamma_1 t} - e^{\gamma_M t}\right) - \frac{d_{M,s}}{\gamma_2}\left(e^{\gamma_2 t} - 1\right) + \frac{d_{M,s}}{\gamma_2 - \gamma_M}\left(e^{\gamma_2 t} - e^{\gamma_M t}\right)\right) \quad \text{(A18)}$$

with $\lim_{t\to\infty} A_{M,s}(t) = \frac{-\sigma k_s A_0}{\gamma_{M,2}} d_{M,s}\left(\frac{1}{\gamma_1} - \frac{1}{\gamma_2}\right) = \frac{-\sigma k_s A_0}{\gamma_{M,2}} d_{M,s} \frac{-(\gamma_1 - \gamma_2)}{d_{P,m} k_s} = \frac{d_{M,s} A_0}{d_{M,m} + d_{M,s}}$

From the sums $A_{P,s}(t) + A_{M,s}(t)$ and $A_{P,m}(t) + A_{M,m}(t)$ one can obtain the amounts of parent plus metabolite in slice and culture medium respectively. This will not be elaborated, but it may be of interest in case of sampling total activity of spiked material.

Appendix B *In vitro-in vivo* extrapolation

In a physiologically based pharmacokinetic (PBPK) model conception analogous to the slice model, the liver compartment is subdivided in two subcompartments: a blood subcompartment of blood filled vessels which exchange mass with a second, tissue sub-compartment. In a slice, the vessels will not be blood filled and their contribution to the total slice volume will be neglected. The typical mass balance equations read:

$$\begin{aligned} \frac{dA_{l,b}}{dt} &= Q_l(C_a - C_{l,b}) - D_l(f_b C_{l,v} - f_{l,t} C_{l,t}) \\ \frac{dA_{l,t}}{dt} &= D_l(f_b C_{l,b} - f_{l,t} C_{l,t}) - K_{l,t} f_{l,t} C_{l,t} \end{aligned} \tag{B1}$$

Here, Q_l is the blood flow through the liver, D_l is a diffusion coefficient for mass exchange between the two subcompartments and $K_{l,t}$ is the intrinsic clearance of the tissue compartment. The first term in the right hand side of line one describes the flow driven mass exchange between blood and the liver blood subcompartment and the second term diffusion driven exchange between the two subcompartments. The last term in the second line represents the intrinsic clearance. Note that the subcompartments are considered as well stirred.

As the liver is well perfused, with only a few cell layers between the arterioles and venules, it is assumed that, in distinction with a slice, diffusion is fast. As a consequence instantaneous equilibrium between the free fractions in blood and tissue will be assumed: $f_b C_{l,b} = f_{l,t} C_{l,t}$. As the concentration in liver $C_l = v_{l,b} C_{l,b} + v_{l,t} C_{l,t}$, where the weighting factors v are the relative sub-compartment volumes, it follows that

$$C_l = (v_{l,b} + \frac{f_b}{f_{l,t}} v_{l,t}) \cdot C_{l,b} = P_l C_{l,b} \tag{B2}$$

and the total amount in the liver can be obtained by summing the two lines of (B1):

$$\frac{dA_l}{dt} = Q_l(C_b - C_l / P_l) - f_b K_{l,t} C_l / P_l \tag{B3}$$

Now, assuming that the specific clearances of the liver slice and the liver tissue subcompartment are the same, i.e., $K_{l,t} / V_{l,t} = K_s / V_s$, the last term in (B3), expressed in terms of the slice clearance K_s shows the required *in vitro-in vivo* extrapolation:

$$\frac{dA_l}{dt} = Q_l(C_b - C_l / P_l) - f_b \frac{V_{l,t}}{V_s} K_s C_l / P_l \tag{B4}$$

The basic assumption is that the free fractions in slice and liver tissue and the specific intrinsic clearances of slice and liver tissue are the same, i.e., the slice is an appropriate model for liver tissue. Note that also the compound's free fraction in blood is involved in the extrapolation:

$f_b / P_l = v_{l,b} / f_b + v_{l,t} / f_{l,t}$, i.e., the harmonic mean of the free fraction in blood and liver tissue, weighted by the relative blood and tissue volume.

Appendix C System model Laplace transformation and identification

Consider the system described in (A1) as a compartment system

$$\dot{\underline{A}} = \underline{\underline{M}}\,\underline{A} \tag{C1}$$

where

$$\underline{A} = \left(A_{P,m},\, A_{P,s},\, A_{M,s},\, A_{M,m}\right)' \tag{C2}$$

and

$$\underline{\underline{M}} = \begin{pmatrix} -d_{P,m} & d_{P,s} & 0 & 0 \\ d_{P,m} & -d_{P,s} - k_s & 0 & 0 \\ 0 & k_s & -d_{M,s} & d_{M,m} \\ 0 & 0 & d_{M,s} & -d_{M,m} \end{pmatrix} \tag{C3}$$

Application of the Laplace transformation $\underline{X}(s) = \int_0^\infty \underline{A}(t) \cdot \exp(-s \cdot t)\, dt$ transforms the system of differential equations (A11) in a system of linear equations

$$\left(s\underline{\underline{I}} - \underline{\underline{M}}\right)\underline{X} = \underline{\underline{B}}\,\underline{A}_0 \tag{C4}$$

where $\underline{\underline{I}}$ is the identity matrix and $\underline{\underline{B}} = \left(1, 0, 0, 0\right)'$ is the 4×1 matrix representing an experiment with initial concentration in culture medium. Were the initial concentration in slice, this matrix would be $\underline{\underline{B}} = \left(0, 1, 0, 0\right)'$.

An observation matrix $\underline{\underline{C}}$ is defined, depending on which (combinations of) compartments are observed. E.g., when the parent compound in slice is observed, then $\underline{\underline{C}} = \left(0, 1, 0, 0\right)$, when the parent compound and the metabolite is observed both from the pool of culture medium and slice, then $\underline{\underline{C}} = \begin{pmatrix} 1, 1, 0, 0 \\ 0, 0, 1, 1 \end{pmatrix}$. This way, one obtains for the vector of Laplace transformed observations

$$\underline{Y} = \underline{\underline{C}}\left(s\underline{\underline{I}} - \underline{\underline{M}}\right)^{-1} \underline{\underline{B}}\,\underline{A}_0 \tag{C5}$$

The coefficients of the so called transfer matrix $\underline{\underline{C}}\left(s\underline{\underline{I}} - \underline{\underline{M}}\right)^{-1} \underline{\underline{B}}$ are rational functions in *s*. he coefficients of these rational functions are algebraic in the system parameters. E.g., when the initial amount is in culture medium and sampling is in the slice, then the rational function is

$$\frac{d_{P,m}}{s^2 + \left(d_{P,m} + d_{p,s} + k_s\right)s + d_{P,m}k_s} \tag{C6}$$

From this function the observables in Laplace space are

$$\varphi_1 = d_{P,m} + d_{P,s} + k_s, \varphi_2 = d_{P,m}k_s, \text{ and } \varphi_3 = d_{P,m} \tag{C7}$$

with as solution

$$d_{P,m} = \varphi_3, k_s = \varphi_2 / \varphi_3 \text{ and } d_{P,s} = \varphi_1 - \varphi_3 - \varphi_2 / \varphi_3 \tag{C8}$$

and thus the system parameters are identifiable from this experiment.
However, when sampling metabolite pooled from slice and culture medium, then the rational function is

$$\frac{d_{P,m}k_s / s}{s^2 + \left(d_{P,m} + d_{p,s} + k_s\right)s + d_{P,m}k_s} \tag{C9}$$

From this function the observables in Laplace space are

$$\varphi_1 = d_{P,m} + d_{P,s} + k_s, \varphi_2 = d_{P,m}k_s = \varphi_3 \tag{C10}$$

and thus the system parameters are not identifiable from this experiment.

Appendix D Sensitivity of concentration time profiles for variation in observable parameters

Approximate solution of parent concentration in slice

The behaviour of the analytic solution for parent concentration in slice (A5) is analysed for various trajectories in time. The results of this analysis will be used for a sensitivity analysis of the parent concentration in slice to variation in the observable parameters.

When $t \approx 0$, then a Taylor series expansion of the function

$$s(t) = \sigma(e^{\gamma_1 t} - e^{\gamma_2 t}) \tag{D1}$$

i.e., $s(t) = A_{P,s}(t) / A_0$, is applied:

$$\begin{aligned} s(t) &\approx \sigma\left((1+\gamma_1 t + \tfrac{1}{2}(\gamma_1 t)^2) - (1+\gamma_2 t + \tfrac{1}{2}(\gamma_2 t)^2)\right) \\ &\approx \sigma\gamma_1 t(1+\tfrac{1}{2}\gamma_1 t) \end{aligned} \tag{D2}$$

where terms with γ_2 have been neglected, because $|\gamma_2| << |\gamma_1|$. So, when $t << 2/|\gamma_1|$, then the parent concentration in slice behaves linear in *t*.

When $t \approx -\ln(2)/\gamma_1$, then

$$s(t) \approx \sigma\left(e^{\gamma_1 t} - 1\right) \tag{D3}$$

and when $1/|\gamma_1| << t << 1/|\gamma_2|$

$$s(t) \approx -\sigma(1+\gamma_2 t) \tag{D4}$$

For still greater values of *t*

$$s(t) \approx -\sigma e^{\gamma_2 t} \tag{D5}$$

Sensitivity of parent concentration in slice to variation of observable parameters

The sensitivity for variations in the observable parameters of the parent concentration in slice is analysed for various trajectories in time. Variation of the parameter with respect to a nominal value will be denoted as follows:

$$p^* = \alpha p$$

where $p = \gamma_1$ or γ_2 or σ. When $\alpha = 1 + \varepsilon$, then it can be considered as representing a small relative error, but in this analysis it can also be considered as a well from 1 deviating factor, say $\frac{1}{2}$ or 2.

When $t << 2/|\gamma_1|$, then $s(t) \approx \sigma\gamma_1 t \approx d_{P,m} t$, and the solution is insensitive to variations of γ_2. The solution is equally sensitive to variations in σ and γ_1. E.g., when $\sigma^* = \alpha\sigma$ then $s^* = \alpha s$. Note however that this does not imply the identification of these two parameters separately, but rather the identification of their product.

When $t = -\ln(2)/\gamma_1$, then $s(t) \approx \sigma(\exp(\gamma_1 t) - 1)$ and the solution is still insensitive to variations of γ_2, while it is equally sensitive to variations in σ as before. When $\gamma_1^* = \alpha\gamma_1$, then

$$\frac{s^*}{s} \approx \frac{2^{-\alpha} - 1}{2^{-1} - 1} = 2 - 2^{1-\alpha} \tag{D6}$$

When e.g. $\alpha = \frac{1}{2}$, then this ratio is about 0.6, and when $\alpha = 2$, this ratio is 1.5. So, this parameter is still sensitive, but less than before.

When $1/|\gamma_1| << t << 1/|\gamma_2|$, then $s(t) \approx -\sigma(1 + \gamma_2 t)$ and the solution has become insensitive for variations in γ_1, while it is equally sensitive for variations in σ as before. When $\gamma_2^* = \alpha\gamma_2$, then

$$\frac{s^*}{s} \approx \frac{1 + \alpha\gamma_2 t}{1 + \gamma_2 t} \approx (\alpha - 1)\gamma_2 t << 1 \tag{D7}$$

So, the sensitivity to variations in this parameter is very small.

When $t = -\ln(2)/\gamma_2$, then $s(t) \approx -\sigma e^{\gamma_2 t}$ and thus the solution is sensitive for variations in σ, while it is insensitive for variations in γ_1. When $\gamma_2^* = \alpha\gamma_2$, then

$$\frac{s^*}{s} = 2^{1-\alpha} \tag{D8}$$

When $\alpha = \frac{1}{2}$, then this ratio is $\sqrt{2}$, when $\alpha = 2$, then it is $\frac{1}{2}$. So, the solution has become sensitive to variations of the terminal rate.

When $1/|\gamma_2| << t$, then the solution is insensitive for variations in the initial rate and sensitive for variations in σ. Depending on the deviation of α from 1, the sensitivity on variations in the terminal rate can be great. E.g., when $\alpha = \frac{1}{2}$, then $s^*/s = \exp(-\frac{1}{2}\gamma_2 t) >> 1$ and when $a = 2$, $s^*/s = \exp(\gamma_2 t) << 1$. Even for small deviations of α from 1 the sensitivity will ultimately become very great.

Approximate solution of parent concentration in culture medium

The behaviour of the analytic solution for parent concentration in culture medium (A8) is analysed for various trajectories in time. The results of this analysis will be used for an analysis of the sensitivity of the parent concentration in culture medium to variation of the observable parameters.

When $t \approx 0$, then a Taylor series expansion of the function

$$r(t) = \sigma\left((1+\frac{\gamma_1}{d_{P,m}})\exp(\gamma_2 t) - (1+\frac{\gamma_2}{d_{P,m}})\exp(\gamma_1 t) \right) \tag{D9}$$

i.e., $r(t) = A_{P,m}(t) / A_0$, is applied.

$$\begin{aligned} r(t) &\approx \sigma\left((1+\gamma_1/d_{P,m})(1+\gamma_2 t) - (1+\gamma_2/d_{P,m})(1+\gamma_1 t + \tfrac{1}{2}(\gamma_1 t)^2) \right) \\ &\approx \sigma\left(1+\gamma_1/d_{P,m} + \gamma_2 t + \gamma_1\gamma_2 t/d_{P,m} \right. \\ &\qquad \left. -1-\gamma_2/d_{P,m} - \gamma_1 t - \gamma_1\gamma_2 t/d_{P,m} - \tfrac{1}{2}(1+\gamma_2/d_{P,m})(\gamma_1 t)^2 \right) \\ &\approx \sigma\left((\gamma_1-\gamma_2)/d_{P,m} - (\gamma_1-\gamma_2)t - \tfrac{1}{2}(1+\gamma_2/d_{P,m})(\gamma_1 t)^2 \right) \\ &= 1 - \sigma\gamma_1 t - \tfrac{1}{2}\sigma(\gamma_1 t)^2 \end{aligned} \tag{D10}$$

So, when $t << 2/|\gamma_2|$, then the parent concentration in culture medium decreases linearly.

When $t \approx -\ln(2)/\gamma_1$, then

$$r(t) \approx \sigma\left(1+\gamma_1/d_{P,m} - (1+\gamma_1/d_{P,m})e^{\gamma_1 t}\right) \approx 1+\sigma(1-e^{\gamma_1 t}) \tag{D11}$$

where $\sigma = d_{P,m}/(\gamma_1-\gamma_2) \approx d_{P,m}/\gamma_1$ has been used, and when $1/|\gamma_1| << t << 1/|\gamma_2|$,

$$r(t) \approx \sigma(1+\gamma_1/d_{P,m})e^{\gamma_2 t} = (1+\sigma)(1+\gamma_2 t) \tag{D12}$$

For still greater values of *t*,

$$r(t) \approx (1+\sigma)\exp(\gamma_2 t) \tag{D13}$$

Sensitivity of parent concentration in culture medium to variation of observable parameters

The sensitivity for variations in the observable parameters of the pooled metabolite concentration is analysed for various trajectories in time.

When $t << 2/|\gamma_1|$, then $r(t) \approx 1-\sigma\gamma_1 t$, and the solution is insensitive to variations in γ_2. If e.g. $\sigma^* = \alpha\sigma$, then

$$\frac{r^*}{r} \approx \frac{1-\alpha\sigma\gamma_1 t}{1-\sigma\gamma_1 t} = 1+\frac{\sigma\gamma_1 t}{1-\sigma\gamma_1 t}(1-\alpha) \approx 1 \tag{D14}$$

when σ does not exceed 1 too much, which it doesn't. From the same argument for the initial rate, one may conclude that the sensitivity of both σ and γ_1 is small. Moreover, only their product can be identified.

When $t = -\ln(2)/\gamma_1$, then $r(t) \approx 1+\sigma(1-e^{\gamma_1 t})$ and the solution is still insensitive for variations in the terminal rate and slightly sensitive for σ. If $\gamma_1^* = \alpha\gamma_1$, then

$$\frac{r^*}{r} \approx \frac{1+\sigma(1-2^{-\alpha})}{1+\sigma(1-2^{-1})} = 1+\frac{\sigma(\frac{1}{2}-2^{-\alpha})}{1+\frac{1}{2}\sigma} \approx 1 \qquad \text{(D15)}$$

when σ is small, which is the case.

When $1/|\gamma_1| << t << 1/|\gamma_2|$, then $r(t) \approx (1+\sigma)(1+\gamma_2 t)$, and the solution has become insensitive for variations in the initial rate. When $\sigma^* = \alpha\sigma$, then

$$\frac{r^*}{r} \approx \frac{1+\alpha\sigma}{1+\sigma} = 1+\frac{\sigma}{1+\sigma}(\alpha-1) \approx 1 \qquad \text{(D16)}$$

and the same result is obtained for $\gamma_2^* = \alpha\gamma_2$, with in (D16) σ replaced by $\gamma_2 t$. So, the sensitivity is small with respect to these two parameters.

When $t = -\ln(2)/\gamma_2$, then $r(t) \approx (1+\sigma)\exp(\gamma_2 t)$ and the solution is insensitive for variations in γ_1 and slightly sensitive for variations in σ. If $\gamma_2^* = \alpha\gamma_2$, then

$$\frac{r^*}{r} = \frac{(1+\sigma)2^{-\alpha}}{(1+\sigma)2^{-1}} = 2^{1-\alpha} \qquad \text{(D17)}$$

so, the sensitivity for the terminal rate has become appreciable: about 1.4 when $\alpha = \frac{1}{2}$ and $\frac{1}{2}$ when $\alpha = 2$.

When $1/|\gamma_1| << t$, then $r(t) \approx (1+\sigma)\exp(\gamma_2 t)$ and the solution is still insensitive for γ_1, slightly sensitive for σ and sensitive for the terminal rate:

$$\frac{r^*}{r} = \exp\big((\alpha-1)\gamma_2 t\big) \qquad \text{(D18)}$$

which can become very great when $\alpha < 1$ and very small when $\alpha > 1$.

Approximate solution of pooled metabolite concentration

The behaviour of the analytic solution for pooled metabolite concentration (A7) is analysed for various trajectories in time. The results of this analysis will be used for a sensitivity analysis of the metabolite concentration to variation of the observable parameters.

When $t \approx 0$, then a Taylor series expansion to the function

$$m(t) = 1-\frac{\gamma_1\exp(\gamma_2 t)-\gamma_2\exp(\gamma_1 t)}{\gamma_1-\gamma_2} \qquad \text{(D19)}$$

i.e., $m(t) = A_M(t)/A_0$, is applied. Note that

$$m^{(n)}(t) = -\frac{\gamma_1\gamma_2^{(n)}\exp(\gamma_2 t) - \gamma_2\gamma_1^{(n)}\exp(\gamma_1 t)}{\gamma_1 - \gamma_2} = \gamma_1\gamma_2\frac{\gamma_1^{(n-1)}\exp(\gamma_1 t) - \gamma_2^{(n-1)}\exp(\gamma_2 t)}{\gamma_1 - \gamma_2} \tag{D20}$$

where $m^{(n)}$ denotes the n^{th} derivative. Thus,

$$\begin{aligned} m(0) = m'(0) = 0 \quad m''(0) = \gamma_1\gamma_2 \\ m^{(n)}(0) = \gamma_1\gamma_2(\gamma_1^{n-2} + \gamma_1^{n-3}\gamma_2 \cdots + \gamma_2^{n-2});\ n \geq 3 \end{aligned} \tag{D21}$$

and

$$m(t) = \sum_n \frac{m^{(n)}(0)}{n!}t = \tfrac{1}{2}\gamma_1\gamma_2 t^2(1 + \tfrac{1}{3}(\gamma_1 + \gamma_2)t + \cdots) \tag{D22}$$

So, neglecting γ_2,

$$m(t) = \tfrac{1}{2}\gamma_1\gamma_2 t^2(1 + \tfrac{1}{3}\gamma_1 t + \cdots) \tag{D23}$$

i.e., quadratic when $t << 3/|\gamma_1|$.
When $1/|\gamma_1| \approx< t << 1/|\gamma_2|$, then

$$m(t) \approx -\frac{\gamma_2}{\gamma_1} - \gamma_2 t \tag{D24}$$

The first term in (D24), however small it may be, offers a far better approximation over a long time trajectory then $-\gamma_2 t$ alone.
For still greater *t*,

$$m(t) \approx 1 - e^{\gamma_2 t} \tag{D25}$$

Sensitivity of pooled metabolite concentration to the observable parameters
The sensitivity of the pooled metabolite concentration to variations of the observable parameters is analysed for various trajectories in time. As the solution for the metabolite concentration does not contain the scaling factor σ at all, it is insensitive to this parameter.

When $t << 3/|\gamma_1|$, then $m(t) \approx \tfrac{1}{2}\gamma_1\gamma_2 t^2$, and the solution is equally sensitive to variations in γ_1 and γ_2. Note however that this does not imply the identification of these two parameters separately, but rather the identification of their product.

When $1/|\gamma_1| \approx< t << 1/|\gamma_2|$, then $m(t) \approx -\gamma_2/\gamma_1 - \gamma_2 t$, and the solution is still sensitive to variation in the terminal rate. When $\gamma_1^* = \alpha\gamma_1$, then

$$\frac{m^*}{m} \approx \frac{\gamma_2/\alpha\gamma_1 + \gamma_2 t}{\gamma_2/\gamma_1 + \gamma_2 t} = \frac{\alpha^{-1} + \gamma_1 t}{1 + \gamma_1 t} \tag{D26}$$

When e.g. $\alpha = 2$, then this ratio equals $1 - \frac{1}{2}/(1+\gamma_1 t)$ and when $\alpha = \frac{1}{2}$ it equals $1+1/(1+\gamma_1 t)$. So, the sensitivity is not great and becomes smaller for increasing *t*. However, when the greater part of samplings is taken during this time period, the sensitivity is perceptible. In fact, the approximation (D24) was inspired by the observation that the pooled metabolite concentration appeared to be moderately sensitive to variation of the initial rate, while it was expected to be sensitive not at all.

When $1/|\gamma_2| << t$, the solution is insensitive for all observable parameters. This agrees with the fact that ultimately the amount of metabolites equals the initial amount of parent compound A_0.

Appendix E Error propagation from observable to model parameters

Multiplicative errors

In this Appendix, analysis will be performed based on the relation between the model parameters and the observable parameters. Thus the relations in (1) between model parameters and system parameters and the relations in (4) between system parameters and observable parameters will be used. However, in practice always $\gamma_2 << \gamma_1$, because $d_{P,m} << d_{P,s}$, because $V_s << V_m$, the relations in (4) will be approximated by

$$d_{P,m} \approx \sigma\gamma_1 \qquad k_s \approx \gamma_2 / \sigma \qquad d_{P,s} \approx -\gamma_1 - d_{P,m} - k_s \tag{E1}$$

Suppose that the estimation of the observable parameters deviate by some factor from the true value, e.g., due to insensitivity of the parameter value in the sampled time trajectory (see Appendix D). So, $\gamma_1^* = \alpha_1\gamma_1$, $\gamma_2^* = \alpha_2\gamma_2$ and $\sigma^* = \alpha_\sigma\sigma$. Then, in the approximate solution of the system parameters from the observable ones,

$$\begin{aligned} d_{P,m}^* \approx \gamma_1^*\sigma^* \approx \alpha_1\alpha_2 d_{P,m} \quad k_s^* \approx \gamma_2^* / \sigma \approx \frac{\alpha_2}{\alpha_\sigma} k_s \\ d_{P,s}^* + k_s^* \approx -\gamma_1^* \approx \alpha_1 (d_{P,s} + k_s) \end{aligned} \tag{E2}$$

From these equalities follows

$$\begin{aligned} D_P^* \approx \alpha_1\alpha_\sigma D_P \qquad f_{P,s}^* K_s^* \approx \frac{\alpha_2}{\alpha_\sigma} f_{P,s} K_s \\ f_{P,s}^* D_P^* + f_{P,s}^* K_s^* \approx \alpha_1 (f_{P,s} D_P + f_{P,s} K_s) \end{aligned} \tag{E3}$$

The last equality can be solved for $f_{P,s}^*$ by inserting those of the first line in (E3):

$$f_{P,s}^* \approx \left(1 + (1 - \frac{\alpha_2}{\alpha_1\alpha_\sigma}) \frac{K_s}{D_P}\right) \cdot \frac{f_{P,s}}{\alpha_\sigma} \tag{E4}$$

and consequently

$$K_s^* \approx \frac{\alpha_2 K_s}{1 + (1 - \alpha_2 / \alpha_1\alpha_\sigma) \cdot K_s / D_P} \tag{E5}$$

Note that when $\alpha_2 / \alpha_1\alpha_\sigma \approx 1 + D_P / K_s$ the denominator in (E5) will blow up the factor for the slice clearance. Thus the estimated value for the slice clearance will be much greater than the true value, while the corresponding estimate for the compound's free fraction will be much smaller. Also, notice that this condition can be met under two quite different conditions: one

condition is met when sampling is only during a time interval that at least one of the parameters is insensitive, the other is when the ratio of clearance and diffusion parameters is great.

Relative error

Suppose that the observable parameters can be estimated with only a small or moderate relative error not exceeding, say, 10%. Then, with

$$\alpha_1 = 1+\varepsilon_1 \qquad \alpha_2 = 1+\varepsilon_2 \qquad \alpha_\sigma = 1+\varepsilon_\sigma \tag{E6}$$

(E4) and (E5) can be rewritten as

$$f^*_{P,s} \approx \left(1+(\varepsilon_1-\varepsilon_2+\varepsilon_\sigma)\frac{K_s}{D_P}\right)\cdot(1-\varepsilon_\sigma)f_{P,s} \tag{E7}$$

and

$$K^*_s \approx \frac{(1+\varepsilon_2)K_s}{1+(\varepsilon_1-\varepsilon_2+\varepsilon_\sigma)\cdot K_s/D_P} \tag{E8}$$

Note that even if the relative errors are moderate the errors in (E7) and (E8) can become large when $K_s/D_P >\approx 1$. The error in K_s can even blow up for values of $K_s/D_p \approx 1/3|\varepsilon|$, where ε is an overall measure of the relative errors. E.g. when $\varepsilon \approx 10\%$, then errors can blow up if $K_s/D_P \approx 3$.

Appendix F Mailing list

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