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The use of sandwich-cultured rat hepatocytes to determine the intrinsic clearance of compounds with different extraction ratios; 7-ethoxycoumarin and warfarin

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Running title page

In vitro clearance of 7-ethoxycoumarin and warfarin

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CL_{bile}, biliary clearance (ml/min/kg bodyweight); CL_{hep}, hepatic clearance (mL/min/kg bodyweight); CL_{int}, intrinsic clearance (mL/min); C_m, concentration medium (µM); C_s, concentration sandwich (µM); D_m, diffusion coefficient in medium (cm^2/min); D_s, diffusion coefficient in sandwich (cm^2/min); f_a, fraction of cell activity; f_m, free fraction medium; f_h, free fraction hepatocytes; f_n, fraction of cells; f_s, free fraction sandwich; f_u, free fraction in blood; f_v, fraction of viable cells; K_h, specific clearance (-/min); K_l, specific intrinsic clearance of the liver (-/min; -/hr); K_m, Michaelis Menten constant µM; K_{ow}, partition coefficient octanol water; K_s, first order metabolism (-/min); L_m, medium layer thickness (cm); L_s, sandwich layer thickness (cm); PBPK, physiologically-based pharmacokinetics; P_{cm}, collagen-medium partition coefficient; P_{hm}, hepatoycte-medium partition coefficient; P_{sm}, sandwich-medium partition coefficient; P, parameter value; Q_h, hepatic blood flow (L/hr); s, sensitivity; SRW, standard rat bodyweight (250g); V_c, volume collagen (ml); V_h, volume hepatoyctes (ml); V_m, volume medium (ml); Vmax, maxium rat of metabolism (µM/min); V_s, volume sandwich (ml)

Abstract

The application of sandwich-cultured rat hepatocytes for the identification of the hepatic intrinsic clearance of compounds with widely varying extraction ratios was investigated. We previously showed the applicability of this in vitro system, in combination with a model describing molecular diffusion, hepatocyte-medium partition and non saturated metabolism, which resulted in a successful identification of this parameter for tolbutamide. This approach is further validated using the compounds 7-ethoxycoumarin and warfarin, covering a 100-fold range of extraction ratios. Clearance of these two substrates could be reliably determined, but only if the depletion of the parent compound in medium as well as in the hepatocyte sandwich was measured. Sensitivity analyses showed that the time course of depletion of the parent compound in medium, especially for warfarin, is insensitive to the partition and diffusion parameter values, while depletion in the hepatocyte sandwich was far more sensitive. When varying the volumes of collagen in the sandwich culture, it appears that the most reliable kinetic parameters could be obtained by fitting the data with the smaller collagen volume and that these parameters obtained from fitting to data of the larger volumes generally can not be verified satisfactorily with the data of the smaller volumes. The values of hepatic clearance that were obtained after extrapolation of the intrinsic clearance to the hepatic clearance from blood were comparable within a factor of two to hepatic clearance data in the literature. This indicates that this sandwich culture and modeling system can be applied for the identification of the hepatic intrinsic clearance rate of the total range from low to high clearance compounds.

Introduction

Predicting the kinetic parameters of compounds *in vivo* using data from *in vitro* experiments is a fast developing area of research (Obach, 1999; Haenen et al., 2002; Lau et al., 2002; Ito et al., 2004; Ito and Houston, 2004). Intrinsic clearance, which is the key parameter enabling *in vitro-in vivo* extrapolation, can be determined from various *in vitro* models, which include microsomes, liver slices and isolated hepatocytes (including hepatocyte suspensions and plated hepatocytes) (Cross and Bayliss, 2000). A major disadvantage of these models is their short term use, because of declining enzyme activities and viability (Berthiaume et al., 1996; Berry et al., 1997).

Recently we have successfully developed the alternative approach of using sandwich-cultured rat hepatocytes in determining the *in vitro* intrinsic clearance of the slowly metabolized compound tolbutamide (Treijtel et al., 2004). The sandwich culture is a long-term *in vitro* system allowing for long incubation periods. Besides prolonged cell viability other advantages of this system are: cellular morphology comparable to the *in vivo* situation including cellular polarity, physiological levels of protein secretion, development of an extensive bile canalicular network and expression of drug transporter proteins. Furthermore, the most important phase I enzyme activities remain better expressed for several days as compared to hepatocytes cultured without collagen (Koebe et al., 1994; Kern et al., 1997), whereas phase II Glutathione S-transferase activity is stably expressed for over 14 days (Beken et al., 1997b).

In this alternative approach the use of data on substrate depletion was chosen rather than the use of data on metabolite formation. Using data on metabolite formation may cause practical problems when analytical methods or standards to study the kinetics of metabolite formation are not fully optimized. As these problems were confirmed in our previous study with tolbutamide where three unexpected metabolites were detected (Treijtel et al, 2004) the same depletion approach was also chosen in this study. Substrate depletion data obtained from sandwich-cultured rat hepatocytes were incorporated into a mechanistic model. This

model takes into account the system-dependent processes, viz.: molecular diffusion through the medium and collagen layers, protein binding and partitioning to the cell lipid fraction. Ignoring these system-dependent processes could disturb the calculation of the true intrinsic clearance parameter (Treijtel et al., 2004).

The aim of this study was to further develop this promising approach in the prediction of the intrinsic clearance of slowly metabolizing compounds. Moreover, in order to investigate the applicability of the sandwich culture to a possibly wide range of metabolizing kinetics, also the prediction of a fast metabolizing compound was included.

There is a great variety of the collagen layer volume in sandwich cultures that is applied between various studies and laboratories (Berthiaume et al., 1996). Thus another aim was to study the effect of this variation and to understand the limitations the variation of the layer volume imposes. In this perspective the collagen volumes applied in the sandwich culture were varied and the effects of this barrier were studied especially with respect to the difficulty this barrier could impose on gaining accurate experimental data.

Two reference compounds, warfarin and 7-ethoxycoumarin were used in this study, because they cover a wide range (100-fold) of extraction ratios, warfarin being the slow metabolizing compound and 7-ethoxycoumarin the fast metabolizing compound. Moreover, these compounds were widely used in the estimation of kinetic parameters in other *in vitro* systems allowing for comparison of our results with values from the literature. The concentrations used in this study for warfarin and 7-ethoxycoumarin 100 µM and 0.45 µM, respectively, were both below the Michaelis-Menten constant K_m , and the metabolism rate was assumed to be linear.

Once the intrinsic clearance of a compound is determined from an *in vitro* experiment, its value can be used to parameterize an *in vivo* physiologically-based pharmacokinetic (PBPK) model or to estimate the value of the classical kinetic hepatic clearance parameter. For tolbutamide, Treijtel et al. (2004) showed the successful *in vitro-in vivo* extrapolation of the intrinsic hepatocyte clearance that was found experimentally to a PBPK model of the disposition of tolbutamide in the rat (Sugita et al., 1982). In this study, intrinsic clearance of

warfarin and 7-ethoxycoumarin was estimated and extrapolated to a hepatic *in vivo*

clearance.

Materials & Methods

Chemicals

Fetal calf serum and Dulbecco's modified Eagle's medium were purchased from Gibco BRL (Breda, The Netherlands). Insulin, glucagon, hydrocortison, gentamycin, glutathione, bovine serum albumin, collagenase type 1A, testosterone and its metabolites were obtained from Sigma Chemical Co. (St. Louis MO, USA). Collagenase type B was purchased from Roche (Roche Diagnostics Nederland B.V, Almere, the Netherlands). Warfarin and *p*-chlorowarfarin were obtained from Sigma Chemical Co. (Zwijndrecht, the Netherlands) as well as 7-ethoxycoumarin, 10-hydroxycoumarin and methoxycoumarin. All other chemicals were of analytical grade.

Preparation of rat tail tendon collagen

Type I collagen was prepared from rat tail tendons as described by Koebe *et al*. (1994) by a modified procedure of Elsdale and Bard (1972). This preparation yields type I collagen, mostly in its native, not cross-linked, triple-helical form (Beken et al., 1997a).

Isolation of hepatocytes

Male Wistar rats (CPB:uWU) from the Central Animal Laboratory (Utrecht University), weighing 190-250 g were used for hepatocyte isolation. Acidified water and food was provided *ad libitum* before the liver perfusion.

The rats were anesthetized by injecting pentobarbital i.p. (Nembutal®, Ceva Sante Animale, Brussels, Belgium) 120 mg/kg bodyweight. Rat hepatocytes were isolated using the two-step collagenase perfusion technique as described by Seglen (1976) and modified according to Paine *et al*. (1979).

Hepatocyte culture

Collagen gel was prepared by mixing 1 part of DMEM (10x, pH 7.4) and 9 parts of collagen solution of 1.11 mg/ml on ice (yielding a solution of 1.0 mg/ml collagen).The cells were seeded on tissue culture dishes with a diameter of 60mm, precoated with 0.25, 0.50, or 1.0 ml of collagen (underlay), at a density of $4x10^6$ cells/dish in 4 ml of DMEM. DMEM was supplemented with 10% fetal calf serum (FCS), 0.5 U/ml insulin, 0.007 µg/ml glucagon, 7.5 µg/ml of hydrocortisone and 50 µg/ml gentamycin.

After 4 hours of incubation, cultures were washed twice with DMEM (without FCS) and 0.25, 0.50, and 1.0 ml of collagen was added as a top layer on the dishes (overlay) that were precoated with an equal volume. Cultures were placed in the incubator (37˚C, 5% CO_2) for 1 hour to allow gellation. Subsequently, 4 ml of DMEM (without FCS) was added.

Cell viability and enzyme activities

On each day, cultures incubated with warfarin (100 µM) or 7-ethoxycoumarin (0.45 µM) as for the substrate depletion experiments were tested for viability, cell number and biotransformation capacities. Cell viability was determined by trypan blue exclusion. Testosterone hydroxylation activity was determined according to the method of Wortelboer *et al.* (1990). Cytosolic protein was used to estimate cell number and measured according to Lowry *et al.* (1951) using BSA as a standard.

Kinetic profiles of warfarin and 7-ethoxycoumarin in the sandwich rat hepatocyte culture

Twentyfour hours after cell isolation, sandwich cultures were incubated with 4 ml of 100 µM warfarin or 0.45 µM of 7-ethoxycoumarin in DMEM. For the different volumes of collagen used in our experiments, the actual initial values determined were 104, 100, and 101 µM for warfarin and 0.40, 0.48, and 0.49 µM for 7-ethoxycoumarin for collagen volumes of 0.5, 1.0, and 2.0 ml, respectively. For warfarin after 0, 2, 5, 15, 30, 60, 90, 240, 480, 960, 1440, 1920, 2130, and 2880 minutes of incubation for cultures with a collagen volume of 1.0 and 2.0 ml and after 0, 10, 30, 60, 240, 420, 1320, 1500, 1860, and 2990 for sandwich cultures with a

collagen volume of 0.5, a culture was terminated. For 7-ethoxycoumarin after 0, 1, 2, 4, 6, 10, 15, 25, 40, 60, 90, 120, 180, 270, 360, and 480 minutes of incubation a culture was terminated. The medium was aspirated and 2 ml were transferred to a glass centrifuge tube. The sandwich, i.e. the total of collagen and hepatocyte layers was transferred to a glass centrifuge tube as well. All the tubes were ice-cold and stored at −20°C immediately after harvesting. The experiments were carried out in duplicate and n=2.

Analysis of warfarin and 7-ethoxycoumarin

The sandwich samples were thawed and 1.5 ml of a collagenase-Krebs solution (100U/ml) was added. The samples were incubated at 37°C for 60 minutes after which all collagen was digested. 40 μl of 10% acetic acid and 20 μl β-glucuronidase was added to the 7-ethoxycoumarin samples. The samples were incubated at 37ºC for 20 hours to allow the phase II products formed during the kinetic experiments to be deconjugated to hydroxycoumarin. 50 μl of an internal standard solution of *p*-chlorowarfarin (80 ng/ml in acetonitrile) was added to the warfarin samples and 10 μl of methoxycoumarin (80 μM in methanol) was added to the 7-ethoxycoumarin samples. Subsequently, the sandwich samples and the medium samples were extracted with 8 ml of a mixture consisting of dieethylether/dichloromethane/iso-propanol in a 60:40:1 ratio (v/v). After separation and evaporation of the organic layer, the residue of the warfarin samples was dissolved in eluent (acetonitrile and H_2O/H_3PO_4, pH2.7;1:2.4) and the residue of 7-ethoxycoumarin samples was dissolved in 50% methanol (v/v). Calibration curves of warfarin covered a concentration range of 0-150 μM in both medium and sandwich matrix. This concentration range was linear and the limit of detection was 2 μM. For 7-ethoxycoumarin and hydroxycoumarin linear calibration curves covered a concentration range of 0-1.5 μM in both matrices with a limit of detection of 0.025 μM.

HPLC analysis

Twenty µl of the warfarin samples was injected into an HPLC system consisting of a Chromosphere C18 analytical column (100 x 3 mm), with a mobile phase of 0.5% phosphoric acid/acetonitril (63:37, v/v), at a flow rate of 1ml/min. The eluent was monitored with a fluorimetric detector operating at a wavelength of 313 nm for excitation and a wavelength of 400 nm for emission.

7-Ethoxycoumarin and hydroxycoumarin were separated by an HPLC method with fluorimetric detection, slightly modified from Yamazaki (1999). A volume of 10 µl was injected into the HPLC system consisting of a Chromosphere C18 column (100 x 3 mm). The mobile phase consisted of mobile phase A (methanol:milliQ, 25:75, v/v) and mobile phase B (methanol:acetonitril:deionized water, 64:6:30, v/v). Initially, 85% of mobile phase A and 15% of mobile phase B was used. At 3.6 minutes 50% of mobile phase A and 50% of mobile phase B was used and at 11.0 minutes the gradient returned to 85% A and 15% B and re-equilibrated after 9 minutes. The eluent was monitored with a fluorimetric detector operating at a wavelength of 338 nm for excitation and a wavelength of 458 nm for emission.

Mathematical model

Three processes determine the time course of a parent compound in the medium-sandwich experimental model: transport, partitioning and biotransformation. The interplay of these processes typically leads to a biphasic concentration-time course both in medium and the sandwich culture. During the first phase, the concentration in medium rapidly decreases by uptake into the sandwich. The characteristic time of this phase is the sum of the characteristic times of diffusion and biotransformation. The terminal phase is characterized by exponential depletion both in medium and sandwich culture. The exponential rate of this phase, which can be observed from the experiment, multiplied by the medium volume or sandwich culture volume, determines the clearance from medium or sandwich, respectively. Just as, e.g., the hepatic clearance from blood is determined by the liver's intrinsic clearance (biotransformation), the blood free fraction (partition) and the blood volumic flow through the

liver (transport), the clearance from medium is determined by the hepatocyte's intrinsic clearance, the ratio of distribution volume of medium and of the medium-sandwich culture volume of distribution and molecular diffusion. In the appendix, the influence of diffusion and distribution volume on the clearance from medium is shown. When the parameter values neither for partition nor for diffusion are known, it is necessary from an experimental point of view to sample not only during the terminal phase, but also during the initial phase of distribution. The interpretation of data, i.e., the identification of the hepatocyte's intrinsic clearance, necessitates the mathematical formulation of the depletion experiment, its implementation in a computer program and fitting the unknown values of the parameters for clearance, partition and diffusion the experimental data.

Transport takes place by molecular diffusion both in medium and sandwich. Based on our experience with tolbutamide, it is assumed that the values of the molecular diffusion coefficient in medium and sandwich are the same.

Partitioning between the different constituents of the sandwich culture, i.e., medium, collagen and hepatocytes, is assumed to be caused by differences in their protein binding capacity and their lipid content. As neither medium nor collagen contains binding proteins or a lipid fraction the partitioning between these two phases is assumed to be 1. The hepatocytes contain both binding proteins and a lipid fraction.

In the model, the clearance per unit volume of hepatocytes is parameterized, which allows for a straightforward *in vitro-in vivo* extrapolation once the relative volume of hepatocytes in the liver is known. To account for the decrease in cell number, cell variability and biotransformation capacity *in vitro*, the true clearance parameter is corrected by multiplying with the corresponding fractions found, i.e., in the computer model the specific clearance $f_n f_v f_a K_h$ is the product of the true intrinsic specific clearance K_h with the correction factors for fraction of cells (f_n), fraction of viable cells (f_v) and fraction of cell activity (f_a).

The mathematical model description, including the modification with respect to the partitioning of the model in Treijtel *et al.* (2004) can be found in the Appendix. (In the model

equations in Treijtel *et al.*(2004), the division sign, /, between the concentration in sandwich and the sandwich:medium partition coefficient is omitted.) The model parameters for transport (D_m), partition between hepatocytes and medium (P_{hm}) and specific intrinsic hepatocyte clearance, i.e., the intrinsic clearance per unit of volume of hepatocytes, ($K_{int,h}$) were identified by fitting them to the sampling data in medium and sandwich. The volume of hepatocytes was estimated from the number of cells in the culture (4×10^6) and cell volume (7500 μm^3; cell diameter about 24 μm). The fitting criterion was the optimization of the loglikelihood of the set of parameters. Model calculations and model fits were performed using the ACSL-Optimize suite.

In vitro-in vivo extrapolation

For 7-ethoxycoumarin and warfarin, the specific intrinsic hepatocyte clearance was extrapolated to the intrinsic liver clearance by accounting for the volume fraction of hepatocytes in the rat liver (about 90%) and rat liver volume. Next, this estimate of the intrinsic liver clearance was used for an estimation of the hepatic clearance of the compounds, using both a well-stirred and a parallel tube liver model (Wilkinson, 1987).

The well-stirred model assumes instantaneous and complete mixing and reads

$$CL_h = \frac{Q_h f_u CL_{int}}{Q_h + f_u CL_{int}} \tag{1}$$

Here, CL_h is the hepatic clearance from blood, Q_h is the hepatic blood flow and f_u is the free fraction in blood. The parallel-tube model assumes piston flow through parallel tubes causing a concentration gradient in the blood flow direction because of loss due to clearance in the lateral direction and reads

$$CL_h = Q_h \left(1 - \exp\left(\frac{-f_u CL_{int}}{Q_h} \right) \right) \tag{2}$$

Note that when $f_u CL_{int}$ becomes small compared to the blood flow Qh, both models

converge to the same value: $Q_h = f_u CL_{int}$.

Rat cardiac output (110 ml/min), relative hepatic blood flow (18.3%), relative liver volume

(3.4%) were taken from Brown et al. (1997). Standard rat weight is 250 g and the tissues

specific density was taken to be 1. The free fraction of 0.22 for 7-ethoxycoumarin and of

0.017 for warfarin (mean of 0.023 for R-warfarin and 0.011 for S-warfarin) was taken from Ito

and Houston (Ito and Houston, 2004).

Parameter sensitivity analysis

In order to obtain more insight in the reliability of the parameter values that are obtained by

fitting the model to the experimental data, parameter sensitivity was analyzed. This sensitivity

concerns the relative variation in the concentration-time course in medium and sandwich due

to a relative variation in a parameter value:

$$s(t; p) = \frac{p}{C(t; p)} \frac{\partial C(t; p)}{\partial p} \tag{3}$$

Here, *s* is the relative sensitivity, *t* is time, *p* is a generic parameter representing specific

clearance, partition or diffusion and *C* is the concentration in medium or sandwich. From this

expression follows,

$$\frac{\Delta C(t; p)}{C(t; p)} = s(t; p) \frac{\Delta p}{p} \tag{4}$$

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So, at any time *t*, the relative sensitivity at that time point relates a relative variation in the parameter value of interest to the corresponding relative change in the concentration. Samples taken at time points where the sensitivity $s(t)$ is small for the given parameter *p*, poorly contribute to the identification of that parameter; whereas samples taken at time points where the sensitivity is large contribute well to the parameters identification. As the sensitivity depends on the value of the nominal parameters too, i.e., varying the value of the nominal parameters leads to a variety of sensitivities, one can only check the appropriateness of sampling strategy *a posteriori*, unless one knows a fair estimate of the nominal values *a priori*.

Results

Cell number, cell viability, enzyme activity, and cell volume

The results of the following measurements were used for the correction factors (f_n, f_v and f_a)

of the clearance parameter as is described in the Materials and Methods section.

Cell number was estimated by protein determination and decreased from 100% at day one,

to 80% at day 2 and 70% at day 3. Cell viability decreased only slightly: 90% at day 1 and

80% at day 2 and 3. Phase I activity decreased significantly over time. As for both 7-

ethoxycoumarin and warfarin different cytochrome P450 enzymes are involved (Yamazaki et

al., 1996; Ngui et al., 2001; Takahashi and Echizen, 2003). The clearance parameter was

corrected for the mean decrease in activity of the subtypes measured. A decrease of

approximately 50% after four hours of isolation was observed, after which it further

decreased to 40 % at day one and two, until 25% on the third day after isolation. Assuming a

hepatocyte cell diameter of 25 μm, the total cell volume of the hepatocytes plated (about

$4x10^6$ cells) was roughly estimated to be 30 μl.

Substrate depletion of 7-ethoxycoumarin

The experimental data on depletion of 7-ethoxycoumarin in medium and sandwich for

collagen volumes of 0.5, 1.0, and 2.0 ml are shown in figure 1 (left), together with the

corresponding concentration-time course model calculations (log scale was used for the time

axis for a better resolution of the dense sample scheme during the initial phase of the

experiment).

The initial decrease from medium is mostly due to diffusion from medium to sandwich,

resulting in a corresponding initial increase in sandwich. This initial phase is followed by a

terminal phase of decrease in both medium and sandwich due to metabolism. The model

calculations were performed using the result of fitting the model to the data of depletion both

in medium and sandwich and from each individual collagen volume taken together, taking

into account the corresponding collagen volume for each of the three data sets. From a Bayesian statistics point of view this is the better procedure as in this way the individual sets mutually serve as a condition in the fitting procedure. The values found by fitting all data together, resulted in a value for the intrinsic clearance per unit of hepatocyte volume of 6.0 [-/min] (45 µl/min/1x10^6 cells), a value for the hepatocyte-collagen partition coefficient of 29 and a value for the diffusion coefficient of 1.0×10^{-3} [cm^2 / min]

Substrate depletion of warfarin

The experimental data on depletion of warfarin in medium and sandwich for collagen volumes of 0.5, 1.0, and 2.0 ml are shown in figure 1 (right), together with the corresponding concentration-time course model calculations (The same general remarks as for 7-ethoxycoumarin apply). The values found by fitting all data together, resulted in a value for the intrinsic clearance per unit of hepatocyte volume of 0.073 [-/min] (0.55 µl/min/1x10^6 cells), a value for the hepatocyte-collagen partition coefficient of 4.9 and a value for the diffusion coefficient of 0.69×10^{-3} [cm^2 / min]

***In vitro-in vivo* extrapolation**

For 7-ethoxycoumarin the hepatic clearance was estimated to be 6.7 ml/min/SRW = 27 ml/min/kg using the well-stirred liver model and 7.9 ml/min/SRW = 32 ml/min/kg using the parallel tube liver model. For warfarin the hepatic clearance was estimated to be 0.0095 ml/min/SRW = 0.038 ml/min/kg for both the well-stirred liver model and the parallel tube liver model. Table 1 shows both the intrinsic clearance and predicted hepatic clearance of our results compared to the clearances from the literature.

Sensitivity analysis

Sensitivity analysis was performed both for 7-ethoxycoumarin and warfarin using the "sensitivity analysis" option of ACSL-Optimize. The nominal parameter values that were varied, were those obtained by fitting the model to the data. The results for an applied

collagen volume of 0.5 mL are depicted in figure 2 for 7-ethoxycoumarin and figure 3 for warfarin.

The sensitivity to specific intrinsic clearance is always negative, both for the concentration in medium and sandwich, and will increase from the start of the experiment (when no parent compound is at metabolizing sites yet) to the end. The sensitivity to diffusion will always be negative for the concentration in medium: initially because of the faster depletion of the parent compound from medium into sandwich and finally because faster transport to metabolizing sites causes more efficient metabolism. The smaller sensitivity of this parameter in the terminal phase indicates that depletion is limited rather by metabolism than by transport. For the concentration in sandwich, however, initially the sensitivity to diffusion will be positive because of the faster transport from medium into the sandwich, while finally, like in medium, the sensitivity will be negative.

The sensitivity to hepatocyte:medium partition is likewise complex. For this parameter sensitivity for the concentration in medium initially will be negative because of more extended depletion of parent compound, but finally it will be positive because elimination is of the free fraction in the aqueous phase only. So, the greater the partition coefficient, the less effective metabolism will be. However, as in the experiment the total sandwich is sampled, rather than the hepatocytes and collagen layers seperately, the combined partition of the collagen and hepatocyte layers, i.e., the sandwich:medium partition is much smaller than the hepatocyte:medium partition. So, because of this "dilution" of hepatocytes, the sensitivity of the hepatocyte:medium partition for the concentration in medium will always be small compared to the sensitivity of clearance and diffusion. The sensitivity for the concentration in sandwich, however, will always be positive: initially because of the more extended depletion out of the medium and finally because of the less effective metabolism. Moreover, as this parameter determines the concentration in sandwich rather than in medium, it may expect to be more sensitive for the concentration in sandwich than in medium.

Comparing figures 2 and 3, clearly shows the dependency of the sensitivities to the nominal parameter values. Note the differences between 7-ethoxycoumarin and warfarin in the scales of both the time and the sensitivity axes. Also note the differences in sensitivity for warfarin in medium and sandwich in the scale of the y-axis.

Discussion

In a previous study (Treijtel et al., 2004), the clearance estimation of the slow metabolizing compound tolbutamide using a sandwich-culture based approach proved to be useful. From this study it was clear that the specific system parameters for this in vitro approach (viz. the volumes of medium, collagen and cell, the diffusion of compounds in the system as well as the metabolic clearance in the cells) are determining factors in the successful determination of the true intrinsic clearance. Therefore, the in vitro system needed to be complemented with a mathematical modeling approach, taking into account the interplay of these parameters.

Sandwich cultured rat hepatocytes, besides cryopreserved hepatocytes, were also used by Lau et al (2002) to study clearance parameters. However, in their approach the system-dependent parameters that are mentioned above are not taken into account and are therefore obscuring the true intrinsic clearance value. Another difference with our approach lies in the incubation time of only 120 minutes instead of the longer incubation times that were used in our study being the major advantage of using this culture system.

Our approach was further explored with two reference compounds, warfarin and 7-ethoxycoumarin because of their divergent extraction ratio's covering a 100-fold range. Metabolism of warfarin *in vivo* is even slower than tolbutamide (Obach, 1999), whereas 7-ethoxycoumarin, is a very fast metabolizing compound *in vivo* (Carlile et al., 1998). The concentrations used in this study for warfarin and 7-ethoxycoumarin (100 μM and 0.45 μM, respectively) were both below the Michaelis-Menten constant $K_{m,}$. Therefore, metabolism was assumed to be linear. The K_m of 7-ethoxycoumarin was 0.8 μM for hepatocytes (Worboys et al., 1996) and 1.3 μM (Worboys et al., 1995) for liver slices in rat. The K_m of warfarin hydroxylation in fresh rat hepatocytes ranged from 83 μM for 7-OH warfarin formation to 1452 μM for 10-OH warfarin, and from 113 μM for 6-OH warfarin to 339 μM for 10-OH warfarin in cryopreserved hepatocytes (Griffin and Houston, 2004). However, the model could easily be adapted to the case of saturated metabolism.

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For warfarin and 7-ethoxycoumarin a consistent clearance estimation of 0.073/min (45 µl/min/1x10^6 cells) and 6.0/min (0.55 µl/min/1x10^6 cells), respectively, was obtained when fitting on both medium and sandwich data. Model calculations were performed using the result of fitting the model to the data of depletion both in medium and sandwich and from each collagen volume taken together. Due to the relative insensitivity of the concentration-time course in medium to the value of the hepatocyte-collagen partition coefficient, no consistent estimate could be obtained using data of depletion in medium only. When using data of depletion in medium only, then model verification on the sandwich data, using the parameters thus obtained, failed.

For tolbutamide, an *in vivo* physiologically-based pharmacokinetic model was presented by Sugita *et al.* (1982). This model allowed the straightforward *in vitro-in vivo* extrapolation of tolbutamide (Treijtel et al., 2004) with a fair comparison to the experimental data. However, to our knowledge, nor for warfarin, nor for 7-ethoxycoumarin such a model together with experimental data is available. Nevertheless, the parameters resulting from the *in vitro* experiments for 7-ethoxycoumarin and warfarin were extrapolated to the corresponding value for *in vivo* hepatic clearance in blood using the well-stirred and parallel tube liver model and compared to literature values, which were also extrapolated using both liver models. For 7-ethoxycoumarin the hepatic clearance estimated from sandwich-cultured rat hepatocytes were 6.7 and 7.9 ml/min/SRW for the well-stirred and the parallel tube model respectively, while Carlile et al. (1998) estimated a hepatic clearance of 12 ml/min/SRW. Axelsson et al. (2003) estimated a hepatic clearance of 9.8 ml/min/SRW and De Kanter et al. (2004) estimated a hepatic clearance of 3.1 ml/min/SRW. For warfarin, a hepatic clearance of 0.0095 ml/min/SRW was calculated for both models. De Kanter et al. (2002) estimated a hepatic clearance of 0.01 ml/min/SRW. Two important issues should be considered when comparing these literature values which are summarized in table 1. For 7-ethoxycoumarin there is a difference in the clearance based on substrate depletion or metabolite formation such as in Worboys et al. (1995, 1996). For warfarin it is important to use data on the racemic mixture instead of only one of the enantiomers. When comparing the appropriate

literature values which are in accordance (within a factor of two) with the estimated values (table 1), this approach proved to be reliable in clearance estimation.

The sensitivity of the nominal parameters was studied. For ease of phrasing, a parameter will be referred to as sensitive during some time interval, when relative small variations of that parameter will cause relative large deviations in the concentration-time course during that time interval. Data points sampled at time points when a parameter is insensitive will not contribute to its identifiability. In this perspective, the different experimental set-up's with different volumes of collagen applied, was also studied.

With respect to the concentration in medium, the hepatocyte:medium partition is rather insensitive throughout the whole experimental period and the more so for warfarin than for 7-ethoxycoumarin. This explains that when some value for this parameter has been found by fitting to data in medium only, it may happen that verification with respect to the concentration in the hepatocyte sandwich fails. For the concentration in the hepatocyte sandwich the hepatocyte:medium partition is (far) more sensitive. Thus, fitting to data in both medium and hepatocyte sandwich results in a more reliable parameter value estimation. However, when applying greater volumes of collagen, this parameter also becomes insensitive for the concentration in the hepatocyte sandwich, due to increasing dilution of the hepatocytes in the sandwich, which argues for applying the smaller collagen volume. In the appendix (figure A1), a simulation of depletion from medium of a parent compound with the same characteristics but for a tenfold higher hepatocyte:medium partition as 7-ethoxycoumarin is shown. In such a case, i.e., when a drug is lipophilic, not only the apparent clearance is much smaller, but also the sensitivity of the partition is much greater.

Also with respect to the concentration in the hepatocyte sandwich the diffusion parameter appears to be far more sensitive, especially during the initial phase of distribution, than to the concentration in medium. In this case however, the sensitivity increases with increasing collagen volume, due to the longer pathway inside the sandwich to the metabolizing sites. However, in the terminal elimination phase, the sensitivity of the diffusion parameter is at least (for 7-ethoxycoumarin) one order of magnitude smaller than the sensitivity to the

intrinsic clearance. This indicates that the system clearance is rather limited by metabolism, even in the case of the high clearance drug 7-ethoxycoumarin, than by diffusion. In the appendix (figure A1), a simulation of depletion from medium of a parent compound with the same characteristics but for a tenfold lower value of th diffusion parameter as 7-ethoxycoumarin is shown. In such a case, i.e., when the molecular characteristic of the drug prohibits relatively fast transport through medium and the collagen layers, not only the apparent clearance is much smaller, but also the sensitivity of the diffusion is much greater. These simulations stress the importance of taking hepatocyte:medium partition and diffusion, apart from the intrinsic clearance, into account.

During the very initial phase of distribution the intrinsic clearance is rather insensitive, due to the relative small amount of parent compound that has reached the metabolizing sites. But soon, the sensitivity increases with increasing time. Apparently, the sensitivity for warfarin is an order of magnitude lower. However, if one would scale the time for the two drugs corresponding to their half lives, the sensitivities would be similar.

This approach is suited to predict the intrinsic clearance of slow metabolizing compounds such as tolbutamide and warfarin, but can also be used to predict the clearance of fast metabolizing compounds such as 7-ethoxycoumarin. The advantages of using sandwich-cultured rat hepatocytes are the longer incubation periods. This is an important aspect in drug development as most pharmaceutical compounds are low clearance drugs. The fact that this culture system is also suitable for the prediction of high-clearance drugs makes it an all-round system that can be used for the testing of a broad variety of compounds. This is a clear advantage compared with liver slices that do not allow for incubation periods over 24 hours and usually underpredict high-clearance compounds due to diffusional limitations (Worboys et al 1995, 1996).

Thus, the approach described here is suited to predict the intrinsic clearance of slow metabolizing compounds such as tolbutamide and warfarin, but can also be used to predict the clearance of fast metabolizing compounds such as 7-ethoxycoumarin

Acknowledgements

References

Axelsson H, Granhall C, Floby E, Jaksch Y, Svedling M and Sohlenius-Sternbeck AK (2003) Rates of metabolism of chlorzoxazone, dextromethorphan, 7-ethoxycoumarin, imipramine, quinidine, testosterone and verapamil by fresh and cryopreserved rat liver slices, and some comparisons with microsomes. *Toxicol In Vitro* **17:**481-488.

Baars LG, Schepers MT, Hermans JJ, Dahlmans HJ, Thijssen HH (1990) Enantioselective structure-pharmacokinetic relationships of ring substituted warfarin analogues in the rat. J Pharm Pharmacol **42**(12):861-866.

Beken S, Pauwels M, Pahernik S, Koebe H, Vercruysse A and Rogiers V (1997a) Collagen gel sancwich and immobilization cultures of rat hepatocytes: Problems encountered in expressing glutathione S-transferase activities. *Toxicology in Vitro* **11:**741-752.

Beken S, Tytgat T, Pahernik S, Koebe H, Vercruysse A and Rogiers V (1997b) Cell morphology, albumin secretion and glutathione S-transferase expression in collagen gel sandwich and immobilization cultures of rat hepatocytes. *Toxicology in Vitro* **11:**409-416.

Berry MN, Grivell AR, Grivell MB and Phillips JW (1997) Isolated hepatocytes--past, present and future. *Cell Biol Toxicol* **13:**223-233.

Berthiaume F, Moghe PV, Toner M and Yarmush ML (1996) Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration. *Faseb J* **10:**1471-1484.

Brown RP, Delp MD, Lindstedt SL, Rhomberg LR and Beliles RP (1997) Physiological parameter values for physiologically based pharmacokinetic models. *Toxicol Ind Health* 13:407-484.

Carlile DJ, Stevens AJ, Ashforth EI, Waghela D and Houston JB (1998) In vivo clearance of ethoxycoumarin and its prediction from In vitro systems. Use Of drug depletion and metabolite formation methods in hepatic microsomes and isolated hepatocytes. *Drug Metab Dispos* **26:**216-221.

Cross DM and Bayliss MK (2000) A commentary on the use of hepatocytes in drug metabolism studies during drug discovery and development. *Drug Metab Rev* **32:**219-240.

De Kanter R, Monshouwer M, Draaisma AL, De Jager MH, de Graaf IA, Proost JH, Meijer DK and Groothuis GM (2004) Prediction of whole-body metabolic clearance of drugs through the combined use of slices from rat liver, lung, kidney, small intestine and colon. *Xenobiotica* **34:**229-241.

Elsdale T and Bard J (1972) Collagen substrata for studies on cell behavior. *J Cell Biol* **54:**626-637.

Griffin SJ and Houston JB (2004) Comparison of fresh and cryopreserved rat hepatocyte suspensions for the prediction of in vitro intrinsic clearance. *Drug Metab Dispos* **32:**552-558.

Haenen B, Rompelberg C, van Twillert K, Hamzink M, Dormans J and van Eijkeren J (2002) Utility of rat liver slices to estimate hepatic clearance for application in physiologically based pharmacokinetic modeling: a study with tolbutamide, a compound with low extraction efficiency. *Drug Metab Dispos* **30:**307-313.

Ito K, Brown HS and Houston JB (2004) Database analyses for the prediction of in vivo drug-drug interactions from in vitro data. *Br J Clin Pharmacol* **57:**473-486.

Ito K and Houston JB (2004) Comparison of the use of liver models for predicting drug clearance using in vitro kinetic data from hepatic microsomes and isolated hepatocytes. *Pharm Res* **21:**785-792.

Kern A, Bader A, Pichlmayr R and Sewing KF (1997) Drug metabolism in hepatocyte sandwich cultures of rats and humans. *Biochem Pharmacol* **54:**761-772.

Koebe HG, Pahernik S, Eyer P and Schildberg FW (1994) Collagen gel immobilization: a useful cell culture technique for long-term metabolic studies on human hepatocytes. *Xenobiotica* **24:**95-107.

Lau YY, Sapidou E, Cui X, White RE and Cheng KC (2002) Development of a novel in vitro model to predict hepatic clearance using fresh, cryopreserved, and sandwich-cultured hepatocytes. *Drug Metab Dispos* **30:**1446-1454.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem* **193:**265-275.

Ngui JS, Chen Q, Shou M, Wang RW, Stearns RA, Baillie TA and Tang W (2001) In vitro stimulation of warfarin metabolism by quinidine: increases in the formation of 4'- and 10-hydroxywarfarin. *Drug Metab Dispos* **29:**877-886.

Obach RS (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab Dispos* **27:**1350-1359.

Paine AJ, Williams LJ and Legg RF (1979) Determinants of cytochrome P-450 in liver cell cultures. *The liver: Quantitative aspects of structure and function (Eds. Preisig R and Bircher J)***:**99-109.

Seglen PO (1976) Preparation of isolated rat liver cells. *Methods Cell Biol* **13:**29-83.

Sugita O, Sawada Y, Sugiyama Y, Iga T and Hanano M (1982) Physiologically based pharmacokinetics of drug-drug interaction: a study of tolbutamide-sulfonamide interaction in rats. *J Pharmacokinet Biopharm* **10:**297-316.

Takahashi H and Echizen H (2003) Pharmacogenetics of CYP2C9 and interindividual variability in anticoagulant response to warfarin. *Pharmacogenomics J* **3:**202-214.

Treijtel N, Barendregt A, Freidig AP, Blaauboer BJ and van Eijkeren JC (2004) Modeling the in vitro intrinsic clearance of the slowly metabolized compound tolbutamide determined in sandwich-cultured rat hepatocytes. *Drug Metab Dispos* **32:**884-891.

Wilkinson GR (1987) Clearance approaches in pharmacology. *Pharmacol Rev* **39:**1-47.

Worboys PD, Bradbury A and Houston JB (1995) Kinetics of drug metabolism in rat liver slices. Rates of oxidation of ethoxycoumarin and tolbutamide, examples of high- and low-clearance compounds. *Drug Metab Dispos* **23:**393-397.

Worboys PD, Bradbury A and Houston JB (1996) Kinetics of drug metabolism in rat liver slices. II. Comparison of clearance by liver slices and freshly isolated hepatocytes. *Drug Metab Dispos* **24:**676-681.

Wortelboer HM, de Kruif CA, van Iersel AA, Falke HE, Noordhoek J and Blaauboer BJ (1990) The isoenzyme pattern of cytochrome P450 in rat hepatocytes in primary culture, comparing different enzyme activities in microsomal incubations and in intact monolayers. *Biochem Pharmacol* **40:**2525-2534.

Yamazaki H, Inoue K, Mimura M, Oda Y, Guengerich FP and Shimada T (1996) 7-Ethoxycoumarin O-deethylation catalyzed by cytochromes P450 1A2 and 2E1 in human liver microsomes. *Biochem Pharmacol* **51:**313-319.

Yamazaki H, Tanaka M and Shimada T (1999) Highly sensitive high-performance liquid chromatographic assay for coumarin 7-hydroxylation and 7-ethoxycoumarin O-deethylation by human liver cytochrome P450 enzymes. *J Chromatogr B Biomed Sci Appl* **721:**13-19.

Legends for figures

Figure 1

Concentration-time curves of 7-ethoxycoumarin (left) and warfarin (right) in medium (solid line, closed circles) and sandwich (broken line, open circles) for total collagen volumes of 0.5 (upper panels), 1.0 (middle panels) and 2.0 (lower panels) ml. The lines represent model calculations with the optimized parameter values and the symbols represent experimental values.

Figure 2

Sensitivity of concentration-time course in medium (left) and sandwich (right) of 7-ethoxycoumarin to variation in the values of intrinsic clearance (solid line), hepatocyte:medium partition (dotted, broken lines) and diffusion (broken line) for 0.5 mL of collagen. The asterisks show the sampling time points.

Figure 3

Sensitivity of concentration-time course in medium (left) and sandwich (right) of warfarin to variation in the values of intrinsic clearance (solid line), hepatocyte:medium partition (dotted, broken line) and diffusion (broken line) for 0.5 mL of collagen. The asterisks show the sampling time points.

Figure A1.

Left panel: Variation of diffusion with respect to nominal value. Upper line, molecular diffusion ten times slower; middle line, nominal value; lower line, molecular diffusion ten times faster. Right panel: Variation of hepatocyte:medium partition with respect to nominal value. Finally lower line, partition ten times smaller; finally middle line, nominal value; finally upper line, partition ten times greater.

Tables

Table 1

In vitro intrinsic clearance and hepatic clearance from this study compared to literature data. All data were converted to the units µl/min/1x10^6 cells for the in vitro intrinsic clearance and ml/min/SRW (standard rat weight, 250g) for the in vivo hepatic clearance.

in vitro and in vivo clearance of 7-ethoxycoumarin and warfarin

| Study	*in vitro CL $_{int}$*	*in vivo CL $_h$*	reference
	µl/min/1x10^{6}cells	ml/min/SRW	
7-ethoxycoumarin		well-stirred/parallel tube	
sandwich culture	45	6.7/7.9	this study
liver slices	3.9	0.84/0.86	Worboys et al., 1995
hepatocytes	26.4	4.6/5.1	"
liver slices	-	3	De Kanter et al., 2004
fresh hepatocytes	4.7	1.0/1.0	Axelsson et al., 2003
cryopreserved hepatocytes	4.6	1.0/1.0	"
hepatocytes	98	10.5/13.3	Carlile et al., 1998
in vivo	-	12/ -	"
warfarin			
sandwich culture	0.55	0.0095/0.0095	this study
liver slices	-	0.01	De Kanter et al., 2004
fresh hepatocytes	0.097	0.0017/0.0017	Griffin & Houston, 2004
cryopreserved hepatocytes	0.229	-	"
in vivo (S-warfarin)		0.045	Baars et al., 1990
in vivo (R-warfarin)		0.135	

Figure 1
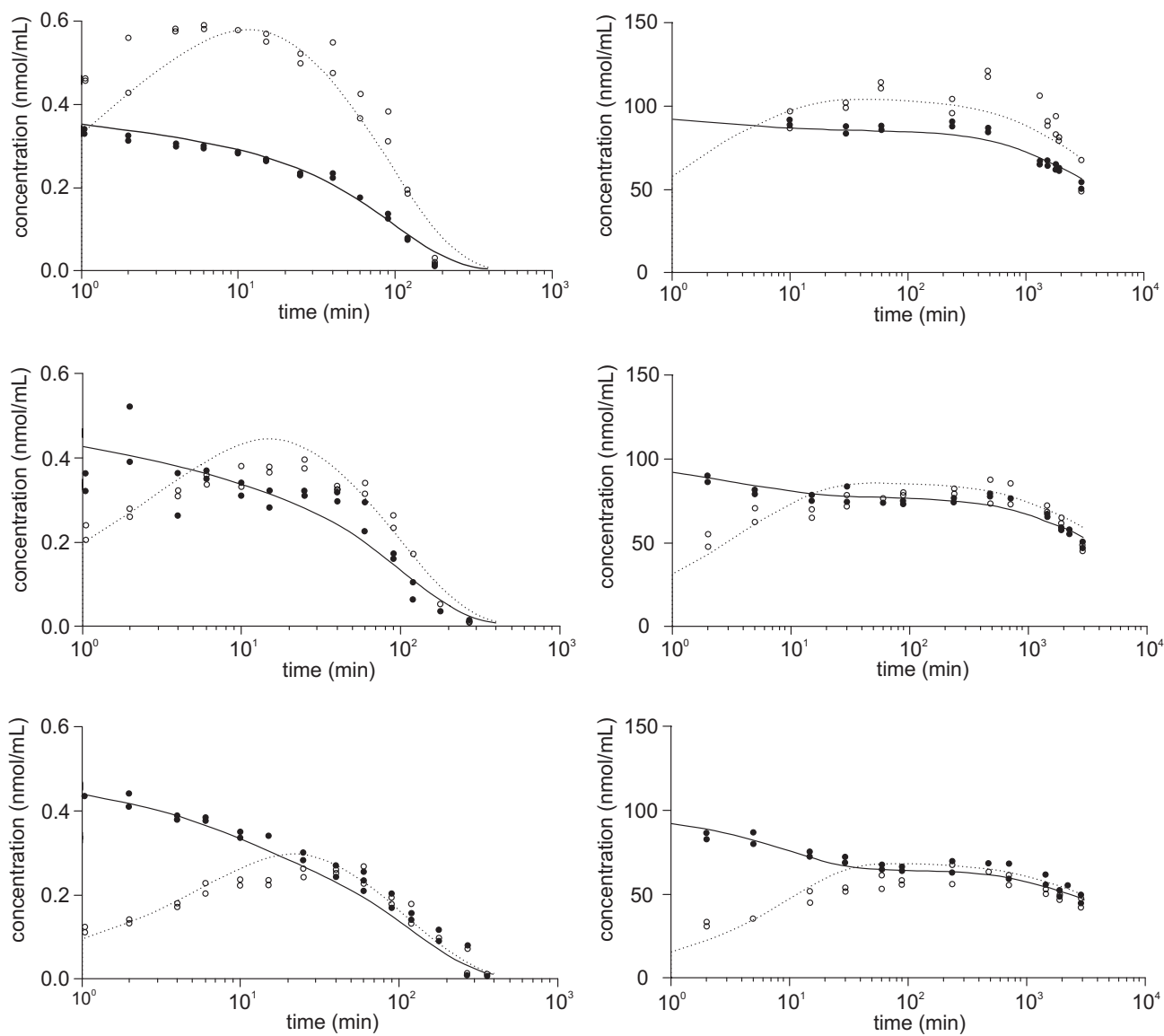
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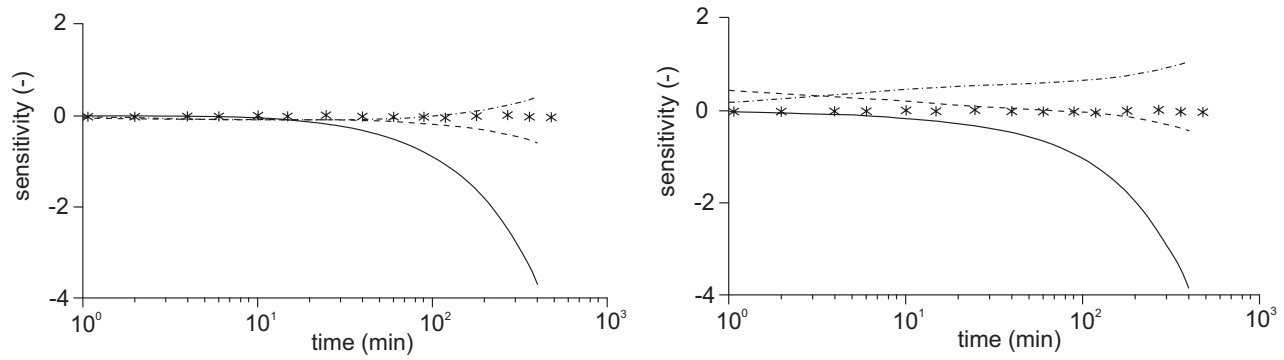
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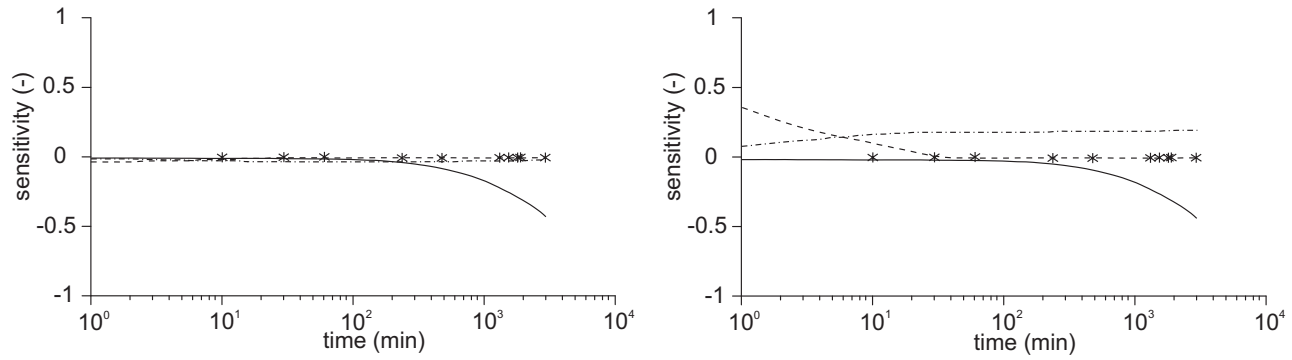
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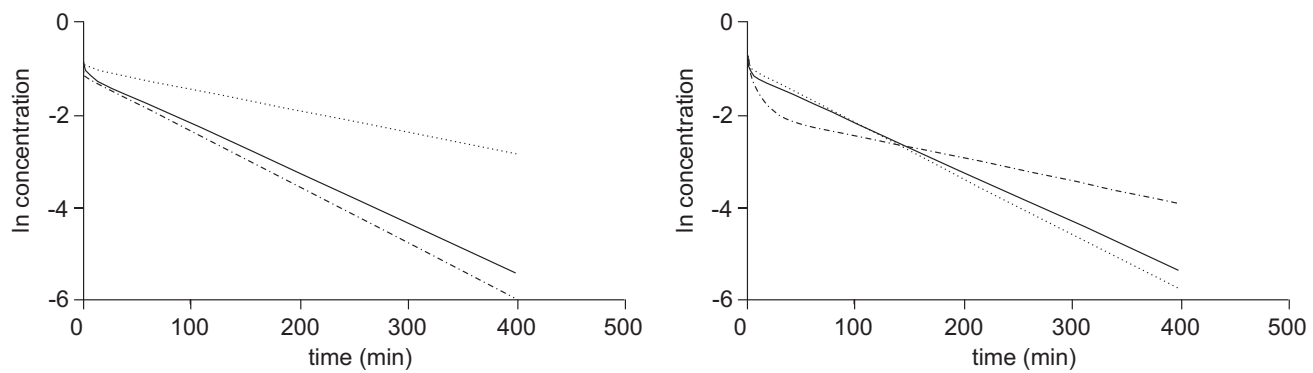
Appendix

In the first section of this appendix, it is shown that both diffusion and partition also determine the exponential rate of depletion. The parameter values for specific clearance, hepatocyte:medium partition and diffusion as found for 7-ethoxycoumarin serve nominal values. In this comparison, it is assumed that a compound differs in its physicochemical properties only with respect to its diffusion (left panel figure A1) or to its partition (right panel figure A1). When one determines the exponential rate from the data and, multiplied by the medium volume, interprets this as the intrinsic clearance, one would conclude to a specific intrinsic clearance of 1.7 ($D_m = 0.1D_m$), 3.9 ($D_m = D_m$,), 4.4 ($D_m = 10D_m$), 4.4 ($P_{hm} = 0.1P_{hm}$), 3.9 ($P_{hm} = P_{hm}$) and 1.8 ($P_{hm} = 10P_{hm}$), while in fact the specific clearance = 6 (-/min).

Unfortunately, the mathematical analysis of diffusion problems is cumbersome. However, when one approximates molecular diffusion by a simple mass transfer between medium and the sandwich culture (which is at least in the initial phase incorrect), then one can derive that the clearance from medium is described by

$$CL_m = \frac{\kappa \cdot V_m / V_d CL_h}{\kappa + V_m / V_d CL_h} \tag{A1}$$

Here, CL_m denotes clearance from medium, κ is the mass transfer coefficient (transport), V_m / V_d is the ratio of the medium and total system distribution volume (partition) and CL_h the hepatocytes intrinsic clearance (biotransformation). The similarity with equation (1) in the main text is obvious. Note that when transport is much faster with respect to clearance, then $CL_m = V_m / V_d CL_h$ and still partition is involved in clearance. In the other extreme case, clearance is much faster, then $CL_m = \kappa$, totally independent of the hepatocyte's clearance.

The model that is described in the text of Materials and Methods with molecular diffusion instead of mass transfer, reads

$$\frac{\partial C_m}{\partial t} = f_m D_m \frac{\partial^2}{\partial x^2} C_m \qquad 0 < x < L_m, \quad 0 < t$$

$$\frac{\partial C_s}{\partial t} = D_s \frac{\partial^2}{\partial x^2} C_s / P_s - K_s C_s / P_s \qquad -L_s < x < 0, \quad 0 < t$$

$$C_m(x,0) = C_{m,0} \qquad C_s(x,0) = 0 \tag{A2}$$

$$f_m D_m \frac{\partial C_m}{\partial x}(0,t) = D_s \frac{\partial C_s / P_s}{\partial x}(0,t) \qquad C_s(0,t)/P_s = f_m C_m(0,t)$$

$$\frac{\partial C_m}{\partial x}(L_m,t) = 0 \qquad \frac{\partial C_s}{\partial x}(L_s,t) = 0$$

The first line of this equation describes transport by diffusion through the medium layer of the free concentration $f_m C_m$. In these experiments the medium (DMEM) contains no binding sites and $f_m = 1$, however, the model can equally well describe culture medium that contains binding sites.

The second line describes the sandwich. While actually this layer is subdivided into three sub-layers consisting of the hepatocyte layer in between two collagen layers, the description assumes a "homogenized" layer with the hepatocytes distributed at random in a layer of collagen. This assumption is only valid when the thickness of the top and bottom layer of the sandwich is the same (Treijtel et al., 2004).

The free concentration that is not dissolved in a lipid fraction is the total concentration C_s divided by the medium:sandwich partition coefficient. As it is assumed that collagen does not contain binding sites, nor has a lipid fraction, $P_s = (V_c + P_h V_h)/(V_c + V_h)$, or equivalently, the distribution volume of the sandwich $P_s(V_c + V_h)$ equals the sum of the distribution volumes of the collagen layers and the layer of hepatocytes $V_c + P_h V_h$. The hepatocyte:medium partition $P_h = P_{hm}/f_h$, where P_{hm} is the lipid/water solubility based hepatocyte:medium partition and f_h is the free fraction in hepatocytes.

The first term in the right hand side of the second line in equation (A2) describes transport by diffusion in the sandwich layer. As the separate identification of the diffusion coefficients in

medium and sandwich is not possible, it assumed that $D_s = D_m$. The second term describes

the clearance per unit of volume $K_s = CL_{int,h}/V_s$, where $CL_{int,h}$ is the intrinsic clearance of

the layer of hepatocytes, $CL_{int,h} = V_h K_{int,h}$ and $K_{int,h}$ is the specific intrinsic clearance of

hepatocytes, i.e., their clearance per unit of volume.

The third line shows the initial conditions: the compound dissolved only in medium with initial

concentration $C_{m,0}$.

The fourth line of equation (A2) describes the conditions at the medium-sandwich interface.

The first equality shows continuity of flux of the compound over the interface, i.e., the

absence of sources or sinks at he interface. The second equality shows instantaneous

equilibration of the free, non lipid soluble fraction of the concentrations at the interface.

The last line shows that there is no transport over the top boundary of the medium layer, nor

over the bottom layer of the sandwich.