MODELING THE IN VITRO INTRINSIC CLEARANCE OF THE SLOWLY METABOLIZED COMPOUND TOLBUTAMIDE DETERMINED IN SANDWICH-CULTURED RAT HEPATOCYTES

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ABSTRACT:

An alternative approach is introduced in determining the in vitro intrinsic clearance of slowly metabolized compounds. The long-term sandwich rat hepatocyte culture was exploited, allowing for sufficient substrate depletion to obtain a reliable clearance estimation; in its physiology, it resembles the in vivo liver, thus allowing in vivo extrapolation of the in vitro clearance value. Substrate depletion of tolbutamide and the formation of its metabolites hydroxytolbutamide and carboxytolbutamide were measured in the medium and sandwich layer. Depletion data from the medium were fitted to a mathematical model incorporating system-dependent parameters (diffusion, protein binding, and partitioning) to calculate the hepatocytes’ intrinsic clearance. Based on the decrease of the parent compound in the medium, a specific intrinsic clearance value, i.e., clearance per unit of volume of hepatocytes, of 0.085 min⁻¹ was fitted. This value was in accordance with in vivo and in vitro values from the literature. The model was verified with substrate depletion data from the sandwich layer. Data on metabolite formation showed an incomplete mass balance. A radiochemical experiment revealed the presence of three additional metabolites. These metabolites were analyzed by liquid chromatography-mass spectrometry. One was identified as p-tolylsulfonyleurea. The structure of the other two needs to be elucidated. After the addition of these compounds to the metabolic pattern, the mass balance was completed. The in vitro clearance value was incorporated in a physiologically based pharmacokinetic literature model of tolbutamide that accurately describes the plasma concentration. The approach used in this study successfully predicts the intrinsic clearance of tolbutamide. In addition, the sandwich rat hepatocyte culture also proves to be useful in the identification of metabolic pathways.

The use of in vitro models to study the kinetics of a compound is of great scientific and practical interest (Worboys et al., 1995; Obach, 1999; Haenen et al., 2002; Lau et al., 2002). Not only does it allow the study of enzyme kinetic mechanisms in more detail, it also provides fast screening methods on biotransformation of compounds. These screening methods can be an important tool in drug discovery as well as in the area of risk assessment. Moreover, these in vitro-derived data can be used in physiologically based pharmacokinetic (PBPK) models to predict the kinetics of a compound in vivo (Houston and Carlile, 1997).

In vitro models are also very useful for determining the intrinsic clearance of compounds. Examples of such in vitro models are microsomes, isolated hepatocytes, and liver slices. A drawback of these models is the time limit imposed on experiments. Enzyme activities drop within a few hours, and in slices and hepatocytes, cell death is observed in less than 6 h (Berthiaume et al., 1996; Berry et al., 1997; Cross and Bayliss, 2000). Even though many successful predictions about kinetics have been made for several compounds, in vitro clearance is still hard to predict for compounds with a low extraction ratio (Iwatsubo et al., 1997; Carlile et al., 1998; Cross and Bayliss, 2000; Naritomi et al., 2001).

The determination of the clearance of a compound requires that substrate depletion during the incubation period is substantial (Obach and Reed-Hagen, 2002; van Eijkeren, 2002). If this cannot be achieved, due to the limitations of the culture system, it is necessary to study the kinetics of substrate uptake by the incubate (slice, hepatocytes, or microsomes) as well as metabolite formation (Haenen et al., 2002; van Eijkeren, 2002). However, sampling the incubate matrix may cause practical problems, and analytical methods or standards to study the kinetics of metabolite formation are often lacking. Especially when drugs are in the early developmental phase and in risk assessment of compounds, this requirement may prohibit its practical use.

The sandwich hepatocyte culture is a long-term culture system that could be exploited to overcome these problems; i.e., it allows for an incubation period that satisfies the requirement of substantial substrate depletion. It consists of hepatocytes plated between two layers of collagen. In this layered matrix, the hepatocytes remain viable for

ABBREVIATIONS: PBPK, physiologically based pharmacokinetic; FCS, fetal calf serum; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high-performance liquid chromatography; RA, radioactivity; LC, liquid chromatography; MS/MS, tandem mass spectrometry; UTB, p-tolylsulfonylurea; ACSL, advanced continuous simulation language.
over a month. Other characteristics of this culture system are a 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 multidrug resistance proteins. Furthermore, phase I enzyme activities remain better expressed for a few days compared with 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., 1997a; Lecluyse et al., 2000). Hence, the clearance of slowly metabolized compounds can easily be determined in this model by measuring the substrate depletion in the medium only.

The determination of the true intrinsic clearance from in vitro cultures, however, may be obscured by system-dependent processes. The true intrinsic clearance is the key parameter to be determined from in vitro data and reflects the pure enzyme activity toward a substrate (Houston and Carlile, 1997). Its estimation should be devoid of parameters that are characteristic of the culture system itself (Haenen et al., 2002; van Eijkeren, 2002). In the sandwich culture, processes such as diffusion in the collagen layers, protein binding, and partitioning to the cell membranes affect the availability of the compound for clearance. If these processes are ignored, only an “apparent” clearance characteristic of the in vitro system is determined. Therefore, the experimental data should be fitted to a mathematical model that describes not only biotransformation but also these system-dependent processes.

In this study, sandwich-cultured rat hepatocytes were used to determine the true intrinsic clearance of the reference compound tolbutamide. Substrate depletion of tolbutamide, which has a low extraction ratio (Haenen et al., 2002), was measured over 72 h. A mathematical model was developed for the culture system to calculate true intrinsic clearance, incorporating diffusion, protein binding, and lipid solubility. The model includes depletion from the medium, substrate uptake by the sandwich, and metabolite formation. For comparison with in vivo data, the in vitro data were extrapolated to an existing PBPK model for tolbutamide in the rat (Sugita et al., 1982).

Materials and Methods

Chemicals. Fetal calf serum (FCS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Insulin, glucagon, hydrocortison, gentamycin, glutathione, bovine serum albumin, collagenase type I A, 1-chloro-2,4-dinitrobenzene, and testosterone and its metabolites were obtained from Sigma-Aldrich. (St. Louis, MO). Collagenase type B was purchased from Boehringer Ingelheim GmbH (Ingelheim, Germany).

Tolbutamide and chloropropamide were obtained from Sigma-Aldrich. Hydroxytolbutamide and carboxytolbutamide were purchased from Brunschwig (Amsterdam, The Netherlands). [14C]tolbutamide with a specific activity of 50 mCi/mmol was obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). 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 the modified procedure of Elsdale and Bard (1972). This preparation yields type I collagen, from rat tail tendons as described by Koebe et al. (1994) by the modified method 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, Utrecht, The Netherlands) weighing 190 to 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, 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. The cells were seeded on 60-mm tissue culture dishes, precoated with 250 µl of collagen, at a density of 4 × 10⁶ cells per dish in 4 ml of DMEM. DMEM was supplemented with 10% FCS, 0.5 µM insulin, 0.007 µg/ml glucagon, 7.5 µg/ml of hydrocortison, and 50 µg/ml gentamycin.

After 4 h of incubation, cultures were washed twice with DMEM (without FCS), and 250 µl of collagen was added as a top layer. Cultures were placed in the incubator (37°C, 5% CO₂) for 1 h to allow gelation. Subsequently, 4 ml of DMEM (without FCS) was added.

Cell Viability and Enzyme Activities. On each day, samples were taken for substrate depletion, and cultures incubated with the same concentration of tolbutamide (225 µM) were tested for viability 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). Cytotoxic protein was used to estimate cell number and measured according to Lowry et al. (1951) using bovine serum albumin as a standard.

Molecular Diffusion Coefficients of Tolbutamide in Medium and Collagen. Culture dishes were coated with two 250-µl layers of collagen without a layer of hepatocytes in between and incubated for at least 1 h after each layer to allow for gelation. The medium (4 ml) containing 225 µM tolbutamide was added to the dishes, which were subsequently incubated until sampled. After 0, 2, 4, 6, 10, 15, 30, 60, and 120 min of incubation, a culture was sacrificed. Medium samples of 2 ml as well as the total amount of collagen were taken. Samples were immediately frozen and stored at −20°C until further analysis.

Kinetic Profiles of Tolbutamide in the Sandwich Rat Hepatocyte Culture. Twenty-four hours after cell isolation, sandwich cultures were incubated with 4 ml of 225 µM tolbutamide. After 0, 3, 6, 9, 12, 15, 20, 30, 45, 60, 90, 120, 180, 360, 540, 360, 540, 720, 1440, 2070, and 2880 min of incubation, a culture was sacrificed. 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 triplicate.

Analysis of Tolbutamide, Hydroxytolbutamide, and Carboxytolbutamide. Samples were analyzed for the parent compound (tolbutamide) and its metabolites (carboxy- and hydroxytolbutamide) by means of a validated, slightly modified HPLC method (Back et al., 1984). In short, the sandwich samples (0.5 ml) were thawed, and 1.5 ml of a collagenase-Krebs’ solution (100U/ml) was added. The samples were incubated at 37°C for 60 min, at which point all collagen was digested. An internal standard solution (100 µl) (200 ng/ml chloropropamide in acetonitrile) and 100 µl of 37% HCl was added. The sandwich and medium samples were subsequently extracted twice with 8 ml of a mixture consisting of diethyl ether/dichloromethane/isopropanol in a 60:40:1 ratio (v/v/v). After evaporation of the organic layer, the residue was dissolved in eluents (acetonitrile and H2O/H₃PO₄, pH 2.7) (1:2.4). Tolbutamide and its metabolites were separated using a Hypersil BDS C₁₈ 3-µm column (Alltech, Breda, The Netherlands) on a Gilson 307 HPLC pump (Gilson International B.V., Rijswijk, The Netherlands) by isocratic elution. Calibration curves of tolbutamide, hydroxytolbutamide, and carboxytolbutamide were made in a concentration range of 0 to 200, 0 to 10, and 0 to 1.5 µg/ml, respectively.

Mass Balance. Kinetic profile experiments showed an incomplete mass balance. To verify that there was no loss of compound from the culture system, experiments with [14C]tolbutamide were performed. 14C-Labeled tolbutamide was mixed with nonlabeled tolbutamide in a ratio of 3:17 to yield a concentration of 225 µM. Sandwich cultures were incubated for 4 and 24 h and treated exactly as they were in the kinetic profile experiments. The mass balance, which was actually the 14C balance, was determined after incubation, after each extraction step, and after dissolving the residue. Radioactivity was measured using Ultra Gold scintillation liquid (PerkinElmer Life and Analytical Sciences, Boston, MA) and a Minaxi Tri-Carb 4000 liquid scintillation counter (PerkinElmer Life and Analytical Sciences). Samples were profiled on an HPLC system (Agilent Technologies, Palo Alto, CA) with a UV and RA detector with online A515 flow scintillation detector.

Metabolite Identification. All photo diode array experiments were performed on a Finngan DECA XP Plus (Thermo Finnigan, San Jose, CA), and all mass spectrometric measurements were performed on a Finnigan TSQ Quantum mass spectrometer equipped with accurate mass option and Surveyor HPLC and autosampler. All mass spectrometric measurements were carried out with electrospray ionization in both positive and negative mode. The isotopic eluents consisted of a mixture of acetonitrile and water (35:65).
adjusted to a pH of 2.7 with formic acid. The sample (20 μl) was injected on the Alltech C18 5-μm (25 × 3.2-mm) column (Alltech). Nitrogen was used as a sheath gas to stabilize the spray.

Mathematical Model. The experimental sandwich culture set up consists of four layers: the medium, the top collagen layer, the hepatocytes, and the bottom collagen layer. Because none of the layers was agitated, they could not be considered to be well stirred.

The layered structure imposes a modeling approach that solves a diffusion problem for each layer with suitable boundary conditions at the medium top layer, the layer interfaces, and the bottom of the bottom collagen layer. Instead, the sandwich itself, i.e., the two collagen layers with the layer of hepatocytes in between, was modeled as only one layer of collagen with a uniform distribution of hepatocytes (Fig. 1). This requires equal upper and lower collagen layer thickness in the experiments, so that the mean path length of diffusion from medium to cells is the same in both models. Comparison of this relative simple two-layer model, also to be interpreted as modeling the “homogenized” sandwich model, with the more complicated four-layer model shows excellent agreement (results not shown).

The model includes transport by diffusion, possible binding of the compound to constituents of the medium, collagen and cells, log (K_m)-based partition between hepatocytes and collagen, and first-order metabolism. The mathematical formulation of the model is as follows:

\[
\frac{dC_m}{dt} = f_s D_m \frac{\partial^2 C_m}{\partial x^2} + \frac{C_m}{V_m} \left( P_m \left( C_m - C_h \right) + C_h \right) \quad 0 < x < L_m, \quad 0 < t
\]

\[
\frac{dC_h}{dt} = f_d D_d \frac{\partial^2 C_h}{\partial x^2} - f_c C_h + f_h C_m \quad L_m < x < 0, \quad 0 < t
\]

\[
C_m(0, x) = C_m(0, 0) = 0
\]

\[
f_s D_m \frac{\partial C_m}{\partial x}(0, t) = f_d D_d \frac{\partial C_h}{\partial x}(0, t) - f_c C_h(0, t) + f_h C_m(0, t)
\]

\[
f_s D_m \frac{\partial C_m}{\partial x}(L_m, t) = f_d D_d \frac{\partial C_h}{\partial x}(L_m, t) = 0
\]

Here, the first line describes the transport of the free fraction (f_m) of compound in the medium (C_m) by diffusion (D_m) with a medium layer thickness (L_m). The second line describes the transport of the free fraction (f_h) of compound in the sandwich (C_h) dissolved in the aqueous phase (P_m) by diffusion (D_d) with a sandwich layer thickness (L_s); it also describes the elimination of the free compound dissolved in the aqueous phase by first-order metabolism (K_h). It is assumed that binding sites are uniformly distributed in both layers and do not contribute to the compounds’ transport. The third line describes the initial conditions in the medium and sandwich, showing the experimental condition of administration of the compound to the medium. The first equation in the fourth line describes the continuity of (free) mass flux over the medium/sandwich interface, whereas the second equation states the steady-state partition between the medium and sandwich at the interface, including log (K_m)-based partition between the sandwich and medium. The last line states the no-mass flow condition at the top of the medium and bottom of the sandwich.

The sandwich medium partition coefficient P_m is obtained from the hepatocyte and collagen medium partition coefficients P_m and P_m, respectively:

\[
P_m = \frac{V_m P_m + V_s P_m}{V_s}
\]

where V_m, V_s and V_i = V_m + V_s denote the volumes of hepatocytes, collagen, and total sandwich, respectively. Equation 2 states that the distribution volume of the sandwich as a whole, P_m V_m, equals the sum of the distribution volumes of hepatocytes and collagen.

Likewise, the free fraction in the sandwich is the harmonic mean of the free fractions in hepatocytes and collagen:

\[
\frac{V_f}{f_f} = \frac{V_s}{f_s} + \frac{V_i}{f_i}
\]

Because the clearance of the homogenized sandwich should be equal to the clearance of the hepatocytes, it follows that the specific clearance K_h, i.e., the clearance per unit of volume of sandwich, is given by the following:

\[
K_h = \frac{V_s}{V_i} K_m
\]

where K_h is the first-order specific intrinsic clearance of the hepatocytes (i.e., the clearance per unit of volume of hepatocytes). In vivo scaling of the specific intrinsic clearance to intrinsic liver clearance is performed by accounting for the weight percentage of hepatocytes in the liver and multiplying with the liver volume. During the experiments, number of cells, cell viability, and phase I enzyme activity were monitored. Fractional corrections for the standard value of the specific clearance allow for the accounting of the decrease in these parameters; i.e., in the computer model, the specific clearance f_n f_d f_c K_h is the product of the true specific intrinsic clearance K_h with the correction factors for fraction of cells (f_n), fraction of viable cells (f_d), and fraction of cell activity (f_c).

The model has been implemented in the versatile ACSL Optimize package (Aegis Software Inc., New York, NY). During parameter fitting, the log likelihood is being optimized.

Lipid Solubility. The octanol-water-based partitioning between the liver slice and medium for tolbutamide was estimated in Haenen et al. (2002) to be P_m = 0.858. This estimation was based on an algorithm for predicting partition coefficients from octanol-water partition coefficient data (Poulin and Krishnan, 1995). The same partitioning is assumed between the hepatocytes and medium. Moreover, the sensitivity of the time course of the concentration in the medium to this parameter is low. Because neither the medium nor collagen contained a fat fraction, the collagen-medium partition coefficient P_cm = 1 was assumed. This was consistent with the experiments regarding the molecular diffusion of tolbutamide.

Free Fraction. Because the medium contained no serum or proteins, the free fraction of tolbutamide in the medium and collagen were assumed to be 1. The free fraction in hepatocytes was fit by Haenen et al. (2002) to data obtained from liver slice experiments. The results, ranging from a fraction of 0.64 to 1, were not conclusive. The model showed that concentrations of the parent compound are insensitive to the value of the free fraction ranging from 0.4 to 1. This can be understood from eq. 3 and the fact that the collagen volume is about 17× the hepatocyte volume. Moreover, fitting this parameter additionally to the specific clearance K_h did not essentially change the likelihood (75.5 compared with 75.3). Therefore, the value f_n = 1 was chosen.

Results

Cell Number, Cell Viability, Enzyme Activity, and Cell Volume.

Cell number was estimated by protein determination and decreased from 100% at day 1, to 80% at day 2, and to 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 shown in Fig. 2. The activity of CYP2C11 (2-alpha testosterone hydroxylation), which is the main enzyme involved in the metabolism of tolbutamide, already decreased to 50% after 4 h of isolation. It only slightly decreased further after 1 and 2 days until 25% on the 3rd day after isolation. Assuming a hepatocyte cell diameter of 25 μm, the total cell
volume of the hepatocytes plated (about $4 \times 10^6$ cells) was roughly estimated to be 30 $\mu$L.

**Molecular Diffusion Coefficients of Tolbutamide.** Figure 3 shows the diffusion of tolbutamide from the medium into collagen in cultures without hepatocytes. Equilibrium was reached after approximately 50 min, at which point an equal concentration was found for the medium and sandwich (partition coefficient equals 1). The mechanistic model was fitted to these data, and the molecular diffusion coefficient was estimated to be 2.7 cm$^2$/min for both the medium and sandwich.

**Tolbutamide Depletion and Metabolite Formation.** The concentration-time course of tolbutamide in the medium and sandwich is shown in Fig. 4. 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 model was fitted through the medium data points, resulting in a $K_{h} = 0.085$ min$^{-1}$. For verification, the tolbutamide concentration in the sandwich was calculated using the same value for the specific hepatocyte clearance obtained from the fit to the medium data. The model represents the data fairly well; only the last few levels seem to be overestimated. The initial decrease of tolbutamide from the medium is mostly due to the diffusion of tolbutamide from the medium to the sandwich, resulting in an initial increase of tolbutamide in the sandwich. This initial phase is followed by a terminal phase of decrease in both the medium and sandwich.

Hydroxytolbutamide formation, which is the primary metabolite, can be observed after 30 min, whereas the secondary metabolite, carboxytolbutamide, is formed after 24 h. Data from the medium and sandwich were pooled, and the total decrease of tolbutamide and total increase of metabolites are shown in Fig. 5. The final recovered amount of metabolites (i.e., hydroxy- and carboxytolbutamide only) was about half of the final decrease in the parent compound.

**Mass Balance.** The experiments with $[^{14}C]$tolbutamide showed a 100% recovery of $^{14}$C after 4 and 24 h. The loss of compound after extraction was negligible, and after dissolving the residue, almost 100% was recovered. HPLC analysis of the samples showed five peaks in the chromatogram (Fig. 6), of which two were identified with authentic standards as tolbutamide and the hydroxytolbutamide metabolite.

**Identification of Metabolites on LC-MS.** The three unidentified compounds in the HPLC-RA chromatograms were analyzed using LC-MS. After a 24-h incubation of the hepatocytes with tolbutamide, four metabolites could be observed. The hydroxytolbutamide metabolite was detected at a retention time of 6.75 min, and the three unidentified metabolites (M1, M2, and M3) were detected at retention times of 5.41, 6.64, and 15.14 min, respectively. M3 could be detected in the mass spectrum in the positive mode; the other metabolites were detected in the negative mode. M1 showed the same fragmentation pattern as tolbutamide after MS/MS (Fig. 7); however, the molecular weight was 56 Da less. This is most likely due to a loss of the C4H9.
chain resulting in the p-tolylsulfonylurea (UTB) metabolite. M2 showed the same fragmentation as M1 and tolbutamide. The molecule weighed 16 Da more than tolbutamide; therefore, this metabolite seems to be oxidized on one of the carbons of the alkyl chain. Measurements of the isotope pattern of M3 showed the presence of sulfur in the molecule. The exact mass was determined to be 243.1482 Da. Using an elemental composition calculator, a molecular formula of C12 H 23 N 2 OS was found to be the most likely possibility. The hypothesized molecular structures are shown in Fig. 8.

In Vitro-in Vivo Extrapolation. Sugita et al. (1982) present a PBPK model for tolbutamide in the rat. We have implemented the model in ACSL, replacing Michaelis-Menten kinetics \( V_{\text{max}} / K_m \) with first-order kinetics. The volume fraction of hepatocytes was assumed to be about 90%. So, the specific intrinsic clearance of the liver was set to be \( K_l / H_1 \approx 0.085 \) min\(^{-1}\). This value was multiplied with liver volume and used for modeling the plasma-concentration time curve, taking all other model parameters as in Sugita’s model.

Figure 9 shows the result together with model calculations using a best fit specific intrinsic clearance value to the plasma data. The figure shows fairly good agreement, demonstrating the practical interest of obtaining in vitro data on in vivo metabolism by sandwich cultures.

Discussion

Various studies have shown that in vitro clearance is hard to predict for slowly metabolizing compounds. Short incubation periods are usually imposed by the in vitro system, and so the amount of substrate depleted is not sufficient for a reliable clearance estimation. Obtaining information on metabolic pathways is not always possible either due to a lack of knowledge or difficulties in the detection of metabolites. Ideally, sampling the parent compound from the culture medium only would be the most advantageous method, because it is easy to perform and analyze (the medium is generally matrix-free). In the present study, these principles were used as the basis for a sandwich culture-based approach in determining the intrinsic clearance of slowly metabolized compounds.

The diabetic drug tolbutamide was chosen as a substrate because of its low extraction ratio and because it is used as a reference compound in many kinetic studies (Sugita et al., 1982; Worboys et al., 1995; Haenen et al., 2002). Furthermore, it enables the comparison with the in vitro method using liver slices (Haenen et al., 2002).

Hepatocytes between two layers of collagen maintain many in vivo characteristics. This resemblance to the in vivo situation is important especially when results are to be extrapolated. Nevertheless, for accurate modeling it was important to monitor some culture characteristics over the same period tolbutamide metabolism was measured. These data were used to correct the calculated intrinsic clearance and involved cell viability, protein content, and enzyme activity. Cell viability and protein content only slightly decreased. For enzyme activity, only the major enzyme responsible for the metabolism of tolbutamide into hydroxytolbutamide, CYP2C11 (Ashforth et al., 1995), was considered. The enzyme activity decreased much faster than viability, reducing metabolizing capacity and showing the necessity of these corrections. Cells cultured after a liver perfusion comprise more than 90% of hepatocytes; therefore, these values were used without further adjustment. Moreover, these values were in agreement with literature values (Beken et al., 1997b; Kern et al., 1997; Lecluyse et al., 2000).

To calculate the intrinsic clearance from the sandwich culture, a mathematical model was developed that incorporates the system parameters of the culture system. Not only does this model include first-order metabolism, it also includes transport by diffusion through the different layers of the system, binding of the compound to constituents of the medium, collagen, cells, and \( K_{\text{ow}} \)-based partition between hepatocytes and collagen. Unlike the classic one-compartment model, this model describes a true intrinsic clearance disposed of these other processes involved. Our four-compartment model consisting of one medium compartment, one cell compartment, and two collagen compartments was simplified to a two-compartment model consisting of one medium and one “sandwich” compartment. This simplification only reduced the amount of parameters and did not change the behavior of the model.

A decrease of tolbutamide at an initial concentration of 225 \( \mu \)M in
the medium culture as well as the formation of metabolites was measured over a 3-day period. Because this initial concentration was still below the $K_m$ of 707 $\mu$M for liver slices and 650 $\mu$M in hepatocytes calculated by Worboys et al. (1995), metabolism was assumed to be linear. This also corresponds with the observation of first-order metabolism kinetics by Haenen et al. (2002), who incubated liver slices in a range of tolbutamide concentrations from 40 to 170 $\mu$M and found no indication for saturation of metabolism. The decrease of tolbutamide in the medium was fitted to the model, taking account of the decrease in cell number, cell viability and activity, and an intrinsic clearance of $0.085$ min$^{-1}$ was obtained. This value was comparable to data from the literature. Haenen et al. (2002) calculated an intrinsic clearance in the range of $0.044$ to $0.110$ min$^{-1}$ after correction for viable cell volume. Contrary to other literature approaches, we succeeded in determining a realistic value with data on the depletion of tolbutamide from the medium alone. In the liver slice-based approach of Haenen et al. (2002), it was also necessary to include kinetic data obtained from sampling the liver slice itself and kinetic data on metabolite formation.

The model was verified using tolbutamide data from the sandwich. This verification showed good agreement to the data. However, when using the data on metabolite formation, the verification failed: the calculated data largely overestimated the experimental data points. This could be explained by an incomplete recovery of the mass balance. Yet, experiments with $^{14}$C-labeled tolbutamide showed a recovery of 100% after an incubation time of 24 h. When observing the metabolite pattern, 10% of the total $^{14}$C activity was recovered as hydroxytolbutamide, a negligible amount as carboxytolbutamide, and 5% as three unknown metabolites. Using mass spectrometry, these metabolites could partially be identified as UTB, tolbutamide hy-
When the estimated in vitro clearance was incorporated in a PBPK model for tolbutamide in the rat, the fit described the data fairly well, considering a different strain of rats was used compared with the study of Sugita et al. (1982). In summary, measuring the concentration of the parent compound in the medium of the sandwich culture was only sufficient to calculate the intrinsic clearance of tolbutamide in contrast to liver slices (Haenen et al., 2002), where data on metabolite formation had to be incorporated as well. Therefore, this culture technique combined with the modeling approach might very well be applicable for other slowly metabolized compounds. Furthermore, the sandwich culture can be used to discover new metabolic pathways that occur after longer incubation times.

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