APPLICATION OF A NEW DYNAMIC MODEL TO PREDICT THE IN VITRO INTRINSIC CLEARANCE OF TOLBUTAMIDE USING RAT MICROSONES ENCAPSULATED IN A FAB HYDROGEL

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Running title: DYNAMIC MODEL TO PREDICT IN VITRO INTRINSIC CLEARANCE OF TOLBUTAMIDE

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ABBREVIATIONS: RLM, rat liver microsomes; FAB hydrogel, F127-acrylamide-bisacrylamide hydrogel; PBPK, physiologically based pharmacokinetic; HPLC, high-performance liquid chromatography; CYP, cytochrome P450 enzyme; NADPH, reduced nicotinamide-adenine dinucleotide phosphate.
Abstract

Currently used in vitro models for estimating liver metabolism do not take into account the physiological structure and blood circulation process of liver tissue. The Bio-PK metabolic system was established as an alternative approach to determine the in vitro intrinsic clearance of the model drug tolbutamide. The system contained a peristaltic pump, recirculating pipeline, reaction chamber, and rat liver microsomes (RLMs) encapsulated in pluronic F127-acrylamide-bisacrylamide (FAB) hydrogel. The metabolism of tolbutamide at initial concentrations of 100, 150, and 200 μM was measured in both the FAB hydrogel and the circular medium. The data from the FAB hydrogel and the circular medium were fitted to a mathematical model to obtain the predicted intrinsic clearance of tolbutamide after different periods of pre-incubation. The in vitro clearance value for tolbutamide was incorporated into Simcyp software and used to predict both the in vivo clearance value and the dynamic process of elimination. The predicted in vivo clearance of tolbutamide was 0.107, 0.087, and 0.095 L/h/kg for intravenous injection and 0.113, 0.095, and 0.107 L/h/kg for oral administration. Compared with the reported in vivo clearance of 0.09 L/h/kg(i.v.) and 0.10 L/h/kg(oral), all the predicted values differed by less than two fold. Thus, the Bio-PK metabolic system is a reliable and general in vitro model, characterized by 3D structured RLM and circulation and perfusion processes for predicting the in vivo intrinsic clearance of low-extraction compounds, making the system more analogous with the rat in terms of both morphology and physiology.
Introduction

It is of scientific and practical significance to use an in vitro metabolism model to study the pharmacokinetics of compounds (Darnell et al., 2012; Lundquist et al., 2014; Ramsden et al., 2014). A metabolite model provides a method for rapidly screening compounds, and is also useful for understanding the mechanisms of metabolism. These screening methods can be used for the discovery of new drugs, risk evaluation, and toxicity predictions (Sengupta et al., 2014). More importantly, the data obtained from these in vitro experiments can be used to generate a physiologically based pharmacokinetic (PBPK) model to forecast the in vivo dynamics of a drug using specifically designed software (Okumu et al., 2009; Willmann et al., 2012; Nordmark et al., 2014).

The currently used in vitro models for studying pharmacokinetics include recombinant enzymes (such as cytochrome P450 and UDPGAT), subcellular fractions (such as microsomes, cytoplasm or S-9 fraction), and cell culture and tissue culture systems (such as hepatic cells and liver slices) (Jia and Liu, 2007). All the in vitro models described above have advantages and disadvantages. The liver slices give an inconsistent metabolic rate because of slow drug transport. The internal cells of the liver slices do not readily come into contact with drugs in the culture solution and the survival of the hepatocytes on the surface of the liver slice is decreased. Although primary hepatocyte cells can simulate in vivo metabolism, they are rapidly inactivated after leaving the body and are difficult to culture (Sahi et al., 2010; Prot et al., 2011). Recombinant enzymes, hepatocytes and microsomes provide more consistent results, but they might not reflect the true in vivo metabolism (Andersson et al., 2012; De Bruyn et al., 2013). All in vitro systems
described above are static, and do not take into account the transport and circulation processes occurring in the liver, which will influence metabolism. At present, none of the conventional *in vitro* methods can simulate the metabolic process with a high degree of accuracy and predictability.

The FAB hydrogel recently invented in our laboratory is a thermosensitive reverse-phase hydrogel, possessing a loose and porous 3D structure suitable for the entrapment of rat liver microsomes (RLM). Microsomes encapsulated in Pluronic F127-acrylamide-bisacrylamide (FAB) hydrogel still retained over 70% metabolic activity (Yang et al., 2013). Herein, we describe the establishment of a new *in vitro* metabolic system consisting of a PTFE tube, peristaltic pump, reaction chamber, and FAB hydrogel. This Bio-PK metabolic system was used to estimate the clearance rate of the model drug tolbutamide. The metabolic reaction together with the convection and diffusion inside the FAB hydrogel were characterized using three groups of experimental data and mathematical simulation to identify the FAB hydrogel metabolic rate constant, CLg. The mathematical model considered depletion from the circular medium, substrate exchange by the FAB hydrogel, circulation, and metabolite formation. For the purpose of comparison with the *in vivo* data, the *in vitro* data were calculated by Simcyp (Simcyp Ltd., Sheffield, UK). This study provides the basis for the development of a more advanced method that can approximate the true physiological situation to reproduce liver metabolism.

Materials and Methods

**Chemicals.** Pluronic F-127, 3, 3′-dioctadecyloxacarbocyanine perchlorate (Vybrant DiO),
tolbutamide, 4-hydroxytolbutamide, dextromethorphan hydro-bromide monohydrate, dextrorphan tartrate, nifedipine, oxidized nifedipine, were purchased from Sigma Aldrich (St. Louis, MO, USA). 30% Acr-Bis (29:1), Ammonium persulfate (APS), and N,N,N’,N’-tetramethylethylenediamine (TEMED) were obtained from the Beyotime Institute of Biotechnology (Nanjing, China). Chlorzoxazone, 6-hydroxy chlorzoxazone and Losartan carboxylic acid were purchased from J&K CHEMICAL (Beijing, China). Losartan potassium was brought from Tokyo Chemical Industry (Tokyo, Japan). Phenacetin, acetaminophen were acquired from Aladdin (Shanghai, China).

**Preparation of RLM.** RLM were prepared as previously described (Nelson et al., 2001). The protein content was quantified by the Lowry method (LOWRY et al., 1951). All animal experiments were carried out in the light of guidelines evaluated and endorsed by the ethics committee of Fudan University.

**Synthesis of F-127’ and FAB hydrogel preparation.** The synthesis of F-127’ and FAB hydrogel preparation was the same as prior methods (Yang et al., 2013).

**The Effective Void Volume of FAB hydrogel.** When the FAB hydrogel were immersed in RLM solutions for 48 h at 4 °C, the volume of one piece of shaped gel was 50μL. To estimate the effective void volume of one piece of FAB hydrogel, 37 shaped and dried FAB hydrogel was weighed before and after immersing in deionized water for 48 h at 4 °C. The void volume could be calculated with the mass difference and density of water. The void volume of FAB hydrogel
was described as $V_g$ as below. As for the final concentration of RLM, we used a formula:

$$v \times c = x \times y$$

Where $v$ was the final medium volume of the whole Bio-PK metabolism system, $c$ was the final working concentration of RLM in circular medium, $x$ was the volume of the effective void volume FAB hydrogel and $y$ was the concentration of RLM contained in the gels, which was equal to the concentration of the RLM immersion solution.

**Release of RLM from FAB Hydrogel in Bio-PK Metabolic System.** To evaluate the FAB hydrogel’s thermosensitivity and encapsulation ability of RLM, FAB hydrogel were immersed in RLM for 48 h at 4 °C to obtain the final concentration of 0.5mg/mL in the medium, which was identical to previous study for comparison between static and dynamic situations (Yang et al., 2013). The gels were then washed within ten seconds in deionized water several times and were put into the reaction chamber of Bio-PK metabolic system at 37°C. The medium in the system was circulated for 24 h with flow speed 6.8mL/min and 50μL circular medium were collected at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h for protein quantitation using the BCA Protein Assay Kit.

**Enzyme Activities and the Impact of Pre-incubation.** In order to compare the metabolic ability between RLM and RLM encapsulated in FAB hydrogel, phenacetin, dextromethorphan, chlorzoxazone, nifedipine and losartan were chosen as the probe drugs for rat CYP 1A2, CYP2D2, CYP 2E1, CYP 3A1/2, CYP 2C11, respectively (Li et al., 2010; Yuan et al., 2010; Tian et al., 2013; Lin et al., 2014a). The metabolic system of FAB hydrogel consisted of the probe drug (final concentrations of 100μM for phenacetin, dextromethorphan, losartan respectively and 200μM for
chlorzoxazone and nifedipine), 1mM NADPH, FAB hydrogel containing RLM to gain final concentration of 0.833 mg/mL and PBS buffer. The entire system was incubated at 37 °C for 24 h with continuous shaking in water-bath. Meanwhile, traditional RLM incubation assays were performed as follows: substrates were pre-incubated with 0.833 mg/mL RLM in PBS for 5 min at 37 °C with one piece of gel and the total volume 360μL in tubes(n=3). Reactions were initiated by the addition of NADPH (1mM) to obtain a final incubation volume of 100μL for control group and 360μL for FAB hydrogel group. Samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h for both groups and stored at -20°C immediately until further processing.

For pre-incubation experiment, all the experimental conditions were the same (final concentrations of probe drugs were 100μM for phenacetin, dextromethorphan, losartan respectively and 200μM for chlorzoxazone and nifedipine). The pre-incubation time was 0, 1, 2, 4h respectively at 37°C, followed by 1 hours’ incubation. Statistical analysis was performed by One-way Analysis of Variance (ANOVA) followed by Tukey post hoc test, using software GraphPad Prism 5.01 (GraphPad Inc.,La Jolla, CA). P<0.05 was considered statistically significant.

**Molecular Diffusion Coefficients of Tolbutamide in Medium and FAB hydrogel.** The medium (0.5 mL) containing 225μM tolbutamide and FAB hydrogel containing 0.3025mg RLM was added to the tube, which were subsequently incubated for 0, 2, 4, 6, 10, 15, 30, 60, and 120 min until sampled. Medium samples as well as the total amount of FAB hydrogel were taken. After sampled, a FAB hydrogel was dissolved by 0.01M NaOH. Samples were immediately frozen and stored at -20°C until further analysis.
Construction of the Bio-PK Metabolic System. The Bio-PK device consisted of a peristaltic pump, polytetrafluoroethylene tube, reaction chamber, and RLM encapsulated in the FAB hydrogel. Each part was connected to guarantee both an effective seal and drug mobility. The medium containing tolbutamide was added to the circular medium in the reaction chamber, and the RLM encapsulated in the FAB hydrogel was also fixed in the reaction chamber in a small perforated tube. Thus, the reaction only took place in the presence of RLM. A schematic of the Bio-PK metabolic system is shown in Fig.1 (A). In light of the previous assumption identified for the rat PBPK model (Gao and Law, 2009), in which the total blood flow consisted of 2/3 venous blood and 1/3 arterial blood, together with rat physiological data (Brown et al., 1997), 6.88 mL/min was chosen to be the simulant liver blood flow for the Bio-PK metabolic system, and the volume of medium was 3.6 mL, which was approximately one-fifth of the rat total blood volume.

Kinetic Profiles of Tolbutamide in Bio-PK Metabolic System. The FAB hydrogel containing 3.025mg RLM together with total 3.6mL circular medium made the final protein concentration 0.833mg/mL. After 5 minutes’ pre-incubation in 37°C constant temperature bath, the circular medium containing 1mM NADPH and respectively 100, 150, 200μM TOL were started to circulate with the speed 6.88mL/min in the Bio-PK metabolic system. Metabolism was assumed to be linear, as the highest concentration was approximately 28% of the calculated Michaelis constant (Worboys et al., 1995). Samples of FAB hydrogel and medium were taken separately at 0, 2, 4, 6, 8, 10, 20, 30, 40, 50, and 60 min incubation and stored at -20°C before processing.
Sample treatment and HPLC conditions. For probe drugs, control groups were terminated by the addition of methanol in the amount of twice the reaction volume, whereas FAB hydrogel group handled separately. The hydrogel samples were blow dried by nitrogen after dissolving in 100μL NaOH (0.01 M) by vortex and HCl was followed for neutralization. After that, the reaction liquid was processed by adding methanol twice the reaction volume. All the samples were measured by HPLC analysis for both substrate and metabolites. 20μL of the supernatant was analyzed by HPLC (Waters, Milford, MA). The detection of dextromethorphan (dextrorphan) and chlorzoxazone (6-hydroxyl chlorzoxazone) followed the HPLC method of Huiying Yang’s (Yang et al., 2013). Samples were analyzed for phenacetin (acetaminophen), nifedipine (oxidized nifedipine), losartan (E-3174) by means of validated HPLC method according to previous studies (Farthing et al., 1997; Niwa et al., 2003; Zhu et al., 2009).

For Bio-PK samples which was composed of FAB hydrogel and circular medium (see Fig.1 (B)), the processing method was different. Hydrogel was processed with 600μL NaOH (0.01M) and then vortex 2 minutes to dissolve the FAB hydrogel. 6μL HCl and twofold methanol was added as soon as dissolution. After another 2 minutes’ vortex, the mixture was dried by nitrogen. While 500μL medium sample was taken and twofold methanol was added before vortex, and then dried by nitrogen, too. All dried samples were dissolved with 120μl mixture of methanol and water (1:1, v/v). After that, samples were centrifuged at 12000r/min for 10 minutes. 50μL of the supernatant was analyzed in HPLC (Waters, Milford, MA). The tolbutamide and 4-OH tolbutamide was separated using a mobile phase of methanol-0.5% acetic acid (60:40, v/v) on a Diamonsil C18(2) (4.6×250mm,5μm) at a flow rate of 1 mL/min and 30°C column temperature with a total retention time of 4.0 min and 7.8 min respectively (233nm). Linearity was verified.
over the range of 0.1-100 μM, and the limit of quantification was 0.1 μM. Intra- and inter-assay variability were both less than 15%.

Mathematical Model.

In the Bio-PK metabolic system, the RLM encapsulated in the FAB hydrogel was incubated in the culture medium containing the substrate at an initial concentration $C_{m,0}$. An overview of the parameters of the model is shown in Fig. 2, in which the most important undefined parameters are shown in red font.

It was assumed that five processes could determine the fate of the substrate: transport by diffusion, possible binding of the substrate to RLM, first-order metabolism, a liquid circulation process, and interchange of the substrate between the FAB hydrogel and the circular medium. The assumption was made that differences in the distribution of a compound between the circular medium and the FAB hydrogel was caused by differences in the compound’s partition into the aqueous, lipid, and protein volumetric fractions, although in this mathematical model, there was no lipid present. The chemical properties of the substrate, in combination with the protein binding, determined its distribution. Generally, the free fraction of a compound was a nonlinear function of its total concentration and can be predicted by a specific method (Allen et al., 1998). In this model, we assumed the concentrations to be smaller than the dissociation constant, so that the free fraction was constant and unrelated to the total concentration of the substrate. Enzyme-catalyzed metabolism is described by Michaelis–Menten kinetics (Michaelis et al., 2011). To simplify the Bio-PK metabolic system model, it was assumed that the concentration levels in the FAB hydrogel are lower than the Michaelis constant for saturation. The mathematical formulation
based on the mass balance of the model is discussed in the results section.

\[
\frac{dC_m(t)V_m}{dt} = D \left( \frac{f_gC_g(t)}{P_{gsm}} - f_mC_m(t) \right) + \left( 1 - \gamma \right)QC_m(t - \tau_1) - \gamma QC_u(t) - QC_m(t) \\
\frac{dC_g(t)V_g}{dt} = -D \left( \frac{f_gC_g(t)}{P_{gsm}} - f_mC_m(t) \right) + \gamma QC_m(t - \tau_1) - CL_gf_gC_g(t) - \gamma QC_u(t - \tau_2) \\
C_m(t) = (1 - \gamma)C_m(t) + \gamma C_g(t) \\
C_u(t)V_m = \left[ C_g(t + \tau_2) - CL_gf_gC_g(t + \tau_2) \right]V_m \\
\frac{A_{MB}(t)}{V_m} = CL_gf_gC_g(t) \\
C_m(t) = \frac{A_m(t)}{V_m} ; C_{gel}(t) = \frac{A_g(t)}{V_g} \\
A_m(0) = A_m(0) ; A_g(0) = 0 ; A_{MB}(0) = 0
\]

All of these equations were built on the basis of mass balance, every parameter was representative: \( A \) was for amount [nmol], \( C \) for concentration [\( \mu M \)], \( V \) for volume [mL], \( D \) for diffusion rate [mL/min] and \( f \) for free fraction. Meanwhile, the subscript \( m \) and \( g \) represents for circular medium and FAB hydrogel. \( C_m \) and \( C_g \) means for the concentration of substrate in circular medium and FAB hydrogel, consistent with \( A_m \) and \( A_g \). \( C_u \) defined the substrate concentration that came from hydrogel by circulation. The amount of metabolites was expressed by \( A_{MB} \). The \( \gamma \) means for the cross-over proportion from the one end of the pipe into the FAB hydrogel. \( Q \) indicated the velocity of flow in peristaltic pump [mL/min]. Assuming that the fluid was plug flow, the time from one end to the other end in the pipeline of the peristaltic pump was \( \tau_1 \) and the time that migrated in the gel was \( \tau_2 \). \( \tau_1 \) was 9.2s when the flow was 6.88mL/min, \( P_{gsm} \) the octanol-water-based medium : hydrogel partition. The model has been implemented in the versatile MATLAB 2010b (MathWorks, Natick, MA). During parameter fitting, the discrete model likelihood was being optimized.
Lipid Solubility. The hydrogel-medium partition coefficient $P_{wm} = 1$ was assumed, because neither the medium, FAB hydrogel nor RLM contained a fat fraction.

Free Fraction. Because the circular medium contained no serum or proteins and all the substrate was completely dissolved, the free fraction of tolbutamide in the medium was assumed to be 1, while the $f_g$ was still unknown. With the help of prediction toolbox in Simcyp (Simcyp Ltd., Sheffield, UK), the predicted range could be determined. The input of microsomal protein concentration was 0.833mg/mL and 10mg/mL to be the minimum and maximal value respectively, which came from the final RLM concentration in circular medium and the immersing RLM concentration. As a result, 0.6 to 0.947 was yield as the reference range.

Accuracy of Prediction. The accuracy of prediction was evaluated from the prediction error (difference between predicted and observed in vivo values) for tolbutamide (Shiran et al., 2006), using the average fold-error as a measure of bias, as discussed by Zuegge et al (Zuegge et al., 2001). If $\text{CL}_{\text{predicted}} > \text{CL}_{\text{observed}}$, then:

$$\text{Fold error} = \frac{\text{CL}(\text{mean})_{\text{predicted}}}{\text{CL}(\text{mean})_{\text{observed}}}$$

Otherwise,

$$\text{Fold error} = \frac{\text{CL}(\text{mean})_{\text{observed}}}{\text{CL}(\text{mean})_{\text{predicted}}}$$

With this test a prediction is considered to be successful if the absolute value of the fold error is 2 or less.
Results:

The Proportion of F-127’ in the FAB Hydrogel. Because the efficiency of the double bond synthesis varied from batch to batch, the proportion of F-127’ in the FAB hydrogel was optimized. Using a few model drugs, we investigated the metabolic capacity, flexibility, and mechanical strength of the FAB hydrogel containing 7.5%, 8%, 8.5%, 9%, 9.5%, and 10% F-127’. A total of 8.5% F-127’ was the best carrier proportion for RLM in terms of both metabolic capability and mechanical strength (data not shown).

Release of RLM from the FAB Hydrogel in the Bio-PK Metabolic System. The protein release profile was determined as shown in Fig.3, after successful fabrication of the RLM-loaded FAB hydrogel. Compared with the control data obtained at 37 °C, 0.3%–10% (Yang et al., 2013), both were smooth and invariant curves under the same conditions. The amount of protein released into the circular medium over 24 h ranged from 0.015±0.001 mg/mL to 0.204±0.003 mg/mL. Additionally, a burst release was observed from 15 min to 30 min at 37 °C, amounting to 0.154±0.009 mg/mL for the 1%–8.5% gel. This could be caused by the rapid and persistent flow of the circular medium as well as the thermal motion at the higher temperature, which resulted in more leakage of the RLM compared with the control group. After the initial burst, the microsomal proteins remained firmly locked inside the gels as observed by the flat curve. The leakage of the microsomal proteins from the Bio-PK group was slightly higher than the control group from 30
min onward and became lower at 24 h, indicating it was stable over a long time period. The newly synthetized hydrogel (37 °C, 0.3%–8.5%) in the Bio-PK metabolic system was capable of retaining RLM even under fast flow conditions.

Determination of $V_{gel}$. Owing to the deficiency of the effective void volume of the hydrogel, the weight-loss method was applied to determine the effective void volume of one piece of gel ($V_{gel}$). A total of 37 pieces of shaped and dried hydrogel were weighed before and after immersion in deionized water. All 37 representative pieces from 5 different batches were selected by eye according to their appearance and incubated in pre-warmed (4 °C) deionized water for 48 h. The cumulative dried weight and wet weight of the 37 pieces of hydrogel was 0.1335 g and 1.2529 g, respectively. By assuming the difference between the wet weight and dried weight was caused by deionized water, using a water density of 1 g/cm³, the $V_{gel}$ was calculated to be 30.25 μL per FAB hydrogel.

Enzymatic Activity. To investigate phase I enzymatic activity, dextromethorphan, phenacetin, chlorzoxazone, nifedipine, and losartan were selected as probe drugs of rat CYP2D2, CYP1A2, CYP2E1, CYP3A1/2, and CYP2C11, respectively (Li et al., 2010; Yuan et al., 2010; Tian et al., 2013; Lin et al., 2014a). These drugs were chosen to comprehensively assess the metabolism of the RLM encapsulated in the hydrogel. The initial substrate concentration was set to be high enough so that it would not be limiting; the concentrations were 100 μM for dextromethorphan, phenacetin and nifedipine, and 200 μM for chlorzoxazone and losartan. Time-dependent metabolism results are shown in Fig.4. The amounts of metabolites produced by traditional
microsomal incubation experiments were used as the positive controls (denoted as traditional RLM). In the first 2 h, the RLM-FAB hydrogel group produced lower levels of metabolites than the traditional RLM. However, this difference decreased with time. At 1 h, the metabolite concentrations produced by the RLM-FAB hydrogel group were 96% (dextromethorphan), 46% (phenacetin), 75% (chlorzoxazone), 66% (nifedipine), and 51% (losartan) of those produced by the traditional RLM group. Over time, the percentage of metabolite formation in the FAB hydrogel and controls continued to increase. At 24 h, the metabolite concentrations produced by the RLM-FAB hydrogel group were 89% (dextromethorphan), 70% (phenacetin), 108% (chlorzoxazone), 84% (nifedipine), and 61% (losartan) of those produced by the traditional RLM group. Compared with the traditional microsomal incubation, use of the FAB hydrogel maintained more than 70% of the RLM activity, except for CYP2C11 (61%). As a result, although the pharmacokinetic behavior of each substrate was different in the traditional RLM group, the RLM-FAB hydrogel group exhibited a longer period prior to saturation for each catalytic reaction. What was noteworthy was that the degradation of metabolites might also be delayed in the FAB hydrogel group, especially for the metabolism of chlorzoxazone and phenacetin.

Effect of Different Pre-incubation Times on the Biotransformation Capacity of FAB Hydrogel. The metabolism of probe drugs of rat CYP2D2, CYP1A2, CYP2E1, CYP3A1/2, and CYP2C11 by FAB hydrogel after different periods of pre-incubation are shown in Fig. 5. Phase I enzyme activity decreased slightly and consistently as the pre-incubation time increased. After a pre-incubation time of 1 h, there was no significant difference in the enzymatic metabolism for each respective probe drug ($p > 0.05$). When the pre-incubation time was prolonged to 2 h, the
enzymatic activity remarkably decreased ($p < 0.05$) compared with the group without pre-incubation, except for CYP2D2 (dextromethorphan). After a 4-h pre-incubation, a significant decrease in enzymatic activity was observed ($p < 0.01$ for all respective CYP groups). The activity of CYP2C11 (E-3174), which was the primary enzyme involved in the metabolism of tolbutamide, was 83.2%, 62.6%, and 28.3% of the control enzymatic activity after 1, 2, and 4 h of pre-incubation in the presence of RLM encapsulated in FAB hydrogel. One hour was chosen to be the reaction time for the Bio-PK metabolic system, to ensure maximum retention of enzymatic activity and linear accumulation of metabolite. Accordingly, because reliable measurement of in vitro rates of metabolism are of great importance for accurate calculation of the intrinsic clearance of the hydrogel, it was decided that FAB hydrogel would be pre-incubated with tolbutamide for only 5 min. Furthermore, for the sake of rectification of experimental data, all time points were investigated for losartan. The amount of losartan that remained was 97.8% after 10 min pre-incubation, 98.2% after 20 min, 95.3% after 30 min, 89% after 40 min, 84.1% after 50 min, and 83.2% after 60 min, assuming no metabolic activity loss before 10 min.

Molecular Diffusion Coefficients of Tolbutamide.

Fig. 6 shows the diffusion of tolbutamide from the medium into FAB hydrogel in medium without RLM in the stationary state. Equilibrium was reached after approximately 10 min, at which point an equal concentration of tolbutamide was found in the medium and the hydrogel (partition coefficient equaled 1). A mechanistic mathematical model was applied to the data, and the diffusion coefficient for tolbutamide was estimated to be 0.036 mL/min for both the medium and the hydrogel. It is evident from the figure that the concentrations of tolbutamide in the two
different mediums did not converge together. The FAB hydrogel is a thermosensitive material, and thus, the volume of the gel would decrease as the temperature increased. This characteristic gave rise to a gap in the tolbutamide concentrations when the distribution process was completed, making the stabilized concentration of tolbutamide in the hydrogel slightly lower than that in the medium.

**Time Course of Tolbutamide Depletion and Metabolite Formation in the Bio-PK Metabolic System.** The concentration–time course of tolbutamide in the medium and FAB hydrogel in the Bio-PK Metabolic System is shown in Fig. 7. The initial decrease of tolbutamide from the medium was mostly because of the diffusion of tolbutamide from the medium to the FAB hydrogel, resulting in an initial increase of tolbutamide in the FAB hydrogel. This initial phase was followed by a terminal phase of decrease in both the medium and the FAB hydrogel. The formation of 4-hydroxytolbutamide, which is the primary metabolite, could be observed as soon as the catalyst was added at 2 min. No secondary metabolite was produced by the RLM. The total amount of metabolites from the medium and hydrogel consistently increased over time as shown in Fig. 8. On account of the fast diffusion and perfusion processes, the amount of tolbutamide in the FAB hydrogel increased faster than that in the stationary state (Fig. 6), as shown in Fig. 9. The model represents the data set very accurately where only a few data points seem to be overestimated.

In this model, the metabolic parameter $\text{CL}_{g}$ describes the intrinsic clearance for the entire hydrogel (containing 3.025 mg RLM). The amount of metabolites could be determined using the percentage of residual metabolic capacity at different times by comparison with the no
pre-incubation groups. All calculations were performed using the MATLAB 2010b package by optimizing the discrete model of the parameter values, considering the experimental data and assuming a relative error model. For verification, the metabolite concentrations in the RLM-FAB hydrogel and circular medium were calculated using a correction factor for the specific RLM clearance obtained from the fit to the specific FAB hydrogel intrinsic clearance data. A correction factor of 0.52 was acquired from the slope ratio of the RLM and RLM-FAB hydrogel groups of CYP2C11 metabolism (losartan) using the same time series because the increase in the metabolite from both the RLM and RLM-FAB hydrogel was linear and the metabolic activity remained unchanged. For extrapolation purposes, we focused on the fitted values of the specific intrinsic clearance for the FAB hydrogel (the intrinsic clearance per minute, mL/min), which were 0.0123 mL/min for the 100 μM incubation, 0.0097 mL/min for the 150 μM incubation, and 0.0107 mL/min for the 200 μM incubation. These values were fitted by applying a correction factor that assumes that inactive RLM have equal metabolizing potency as active RLM. As every milligram of RLM protein was considered to have the same metabolic capacity, we obtained values for the specific intrinsic liver clearance of 4.06, 3.21, and 3.53 μL/min/mg for the incubations of 100, 150, and 200 μM tolbutamide, respectively, which were comparatively smaller than the CL_int of 6.2 μL/min/mg obtained previously (Ashforth et al., 1995a).

Mass Balance. To determine the mass balance, the initial amount of tolbutamide in the medium was calculated by a simultaneous fit of the amount of tolbutamide in the culture medium. The loss of compound during the experimental period was considered to be negligible because the pipeline was made of PTFE (polytetrafluoroethylene) or coated with PTFE on the inner tube wall. PTFE is
an excellent lubricant and should reduce the amount of tobutamidine lost to non-specific binding. Additionally, the high recovery of tobutamidine, 99.2% after 0.5 h and 98.6% 1 h, was further evidence of the low degree of non-specific binding in the experimental system.

**Determination of \( f_g \) and \( f_m \).** The amount of protein leakage from the FAB hydrogel was 7.9 to 15% of the total amount of immersed protein in the FAB hydrogel. Considering the maximum coefficient of variation was 15% for our HPLC method, the observed protein leakage most likely had no measurable consequences on the quantification of the free fractions of tobutamidine and its metabolites, and thus, \( f_m = 1 \) was assumed. Using the prediction toolbox in Simcyp, the \( f_g \) was calculated to range from 0.6 to 0.947, and was simulated as 0.81, 0.83, and 0.75, respectively, for 100, 150, and 200 \( \mu \)M concentrations after fitting the data to the mathematical model in MATLAB. All parameters used in Bio-PK mathematical model was described in Table 1.

**In Vitro to in Vivo Extrapolation.**

The specific clearance of tobutamidine in the hydrogel is a function of the amount of RLM in units of milligrams of protein. Thus, the ratio of the intrinsic clearance to the protein quantity was calculated as 4.06, 3.21, and 3.53 \( \mu \)L/min/mg for the incubations of 100, 150, and 200 \( \mu \)M tobutamidine, respectively. We used the Simcyp software to predict the in vivo clearance in rats according to the same dose, administration route, and duration of study, and then compared the data with previous studies, which were 0.09L/h/kg for intravenous injection and 0.10 L/h/kg for oral administration (Sugita et al., 1982; Sun et al., 2014). The simulant drug plasma concentration–time curve together with the in vivo data for both intravenous injection and oral...
administration are illustrated in Figs. 10 and 11, respectively. The in vivo clearances simulated for tolbutamide were 0.107, 0.087, and 0.095 L/h/kg for intravenous injection and 0.113, 0.095, and 0.107 L/h/kg for oral administration of incubations of 100, 150, and 200 μM tolbutamide.

Figs. 10 and 11 show the results together with those from the model calculations using the three incubation concentrations fitted to the specific intrinsic clearance value from the plasma data obtained for both intravenous injection and oral administration. It is evident from the data that they are all in good agreement. Data from the sandwich model (Treijtel et al., 2004) was also included for comparison purposes because both the sandwich model and the Bio-PK system are 3D in vitro models using TOL as a substrate, and thus the predicted in vivo process could be compared directly. The comparison indicates that the results from the Bio-PK system are more similar to the in vivo data compared with the results obtained for the sandwich model. This result confirmed the practical interest of simulating in vivo metabolism data using the Bio-PK metabolic system.

Accuracy of Prediction. To investigate the fold error in the predicted data, the CL(mean)_{observed} was determined from in vivo data to be 0.09 L/h/kg (Sugita et al., 1982) for intravenous injection and 0.10 L/h/kg (Sun et al., 2014) for oral administration. Thus, the fold error for the CL(mean)_{observed} for intravenous injection was 1.19, 1.03, 1.06; and 1.13, 1.05, 1.07 for oral administration for incubations of 100, 150, and 200 μM tolbutamide, respectively. The Bio-PK metabolic system was identified as being successful at predicting in vivo pharmacokinetic parameters if the fold errors were less than 2.
Discussion

To calculate the FAB hydrogel intrinsic clearance, a mathematical model incorporating the culture system parameters was developed. Compared with the classic static model, our model described true dynamic intrinsic clearance composed of the actual processes involved including transport by diffusion, binding of substrate to RLM, first-order metabolism, liquid circulation processes, and interchange of the substrate between the FAB hydrogel and the circular medium. Using this model, metabolism of tolbutamide at concentrations of 100, 150, and 200 μM in the circular medium was measured. Because these initial concentrations of tolbutamide were all below its reported K_m values for liver slices, hepatocytes (Worboys et al., 1995) and RLM (Brown et al., 2007), metabolism was assumed to be linear.

Generally, tolbutamide was metabolized in the Bio-PK metabolic system within a 60-min period because RLM are typically not active after a 2-h incubation (Projean et al., 2003; Jia and Liu, 2007), and because 60 min ensured a linear increase in the production of metabolites as shown by the pre-incubation results. The characteristic time scale of specific intrinsic clearance in terms of the typical diffusion time scale was also important to consider for this model. The former should be large compared with the latter and, in this study the diffusion time was just a fraction of the incubation time. Thus, the hydrogel was considered to be well-stirred. The diffusion coefficient was 0.036 μL/min, which was approximately 10-fold that obtained for the liver slice metabolism of tolbutamide (Haenen et al., 2002), and much larger than the hydrogel clearance. This indicated that substrate contained in the FAB hydrogel would reach a stable concentration when the diffusion process was complete and metabolism was not limited by the deficiency of substrate or diffusion. As shown in Fig. 9, the amount of tolbutamide in the FAB hydrogel
remained relatively unchanged when the diffusion process was completed. Quick diffusion was an advantage of the FAB hydrogel, as the porous character of the gel provides the possibility for compound dissolved in the liquid to freely pass in and out. In a previous study (van Eijkeren, 2002), it was determined that when liver slice diffusion was rate limiting, determination of the slice intrinsic clearance becomes unreliable. This observation led us to compare the Bio-PK metabolic system with a model that includes intraslice diffusion using a compound possessing high-clearance properties (Haenen et al., 2002). The diabetic drug tolbutamide was chosen as the model substrate because it has been used as a reference compound in many kinetic studies (Sugita et al., 1982; Huang et al., 2014; Lin et al., 2014b) and as a substrate for an in vitro method (Haenen et al., 2002; Treijtel et al., 2004). Though tolbutamide has a low extraction ratio (Schary and Rowland, 1983; Bannoura et al., 1998), in the Bio-PK metabolic system tolbutamide quickly diffused in the hydrogel, the accumulation of metabolite increased linearly with time, the substrate and metabolites were accurately detected and the data could be fitted with a high degree of correlation. Thus, the use of the Bio-PK metabolic system was more broad compared with the use of a liver slice. However, when the low clearance of a compound was the result of a slow diffusion process and a fast metabolism process, this “low-clearance drug” could not be evaluated in the Bio-PK metabolic system.

For precise modeling of the in vitro metabolism data, the RLM-FAB hydrogel metabolic capacity needed to be accurately quantitated. During the comparatively short incubation time (60 min), a slight decrease in the metabolic capacity of the RLM was observed. Only one major P450 enzyme, CYP2C11, was responsible for the metabolism of tolbutamide (Ashforth et al., 1995b). Thus, in the mathematical model, the data were rectified using the enzyme kinetic parameters.
derived from the data for the metabolism of losartan by CYP2C11. By examining only one metabolic reaction, all the factors that influence the enzymatic activity, such as the pre-incubation time and activity loss caused by forming the 3D structure to improve metabolizing potency, could be optimized before entering the parameters into the MATLAB program. As shown in Figs. 7 to 9, the data were not linear with respect to concentration. The intrinsic clearance for the 150 μM incubation was minimal (3.21 μL/min/mg protein), while the intrinsic clearance for the 100 μM incubation was maximal (4.06 μL/min/mg protein), and that for 200 μM was in the middle (3.53 μL/min/mg protein). The in vitro clearance value was incorporated into a physiologically based pharmacokinetic (PBPK) model of tolbutamide in Simcyp software to predict both the in vivo clearance value and the dynamic process of tolbutamide elimination. When adding these intrinsic clearance data into the Simcyp software, all the trial design parameters should be set to the in vivo experiment as reported (i.v. bolus, 80 mg/kg, 8 h (Sugita et al., 1982) and oral, 1 mg/kg, 48 h (Wang, X. et al., 2014)). The predicted in vivo clearance of tolbutamide was 0.107, 0.087, and 0.095 L/h/kg for intravenous injection and 0.113, 0.095, and 0.107 L/h/kg for oral. These values were comparable with data from the literature. As shown in Fig. 10, the intrinsic clearance for tolbutamide using sandwich-cultured rat hepatocytes was 0.085 L/h/kg (Treijtel et al., 2004). There was no significant difference between the results obtained from the sandwich model and those obtained from the Bio-PK metabolic system (fold error were all less than 2).

Compared with other in vitro models, we succeeded in determining representative metabolic parameters by ensuring the system could maintain 3D RLM morphology within a dynamic circulating system driven by a peristaltic pump. Predictions using traditional RLM to the Bio-PK model was improved because of the incorporation of dynamic flow and 3D structure in Bio-PK
metabolic system (shown in Supplemental Data). In the liver slice-based approach (Haenen et al., 2002), a successful two-compartment model was used for the determination of specific intrinsic liver clearance. Although liver slices represent the actual physiological structure of the liver and contain the entire set of metabolic enzymes, the transport of substrate between the liver slice and the medium is slow. Additionally, it is difficult to adequately expose the internal hepatocytes in a liver slice to drugs and the enzymes in the exterior layer of hepatocytes can easily lose their activity. Therefore, liver slices are not well-stirred because of diffusion limitation. These problems can be solved by the use of a sandwich hepatocyte culture, which consists of hepatocytes plated between two layers of collagen to mimic cellular morphology (Treijtel et al., 2004). However, although the reaction time can be extended to a few days, the enzymatic activity in a sandwich hepatocyte system still significantly decreases as the time period of incubation increases. Furthermore, sandwich hepatocyte system needs to be prepared fresh, making the system difficult to handle. Although there were no significant differences in the predicted in vivo clearance from RLM, the sandwich model or the Bio-PK system, the latter was the most suitable for the prediction of the in vivo process (Fig. 10). All conventional in vitro models mentioned are static systems, and do not take into account the transport and circulation processes occurring in the liver, which affects the overall kinetics of liver metabolism. While the Bio-PK metabolic system can be potentially advantageous when predicting PK in special populations concerning the circulation processes, e.g., blood flow, lipid and protein in blood, or constructing Bio-PK/PD model to combine PK and PD in one in vitro model.

During the last decade, microfabrication technology has been extended to microfluidics, the so-called “organ-on-a-chip” technology (Venkatesh and Lipper, 2000; Huh et al., 2012; Esch et al.,
2015). *In vitro* models sharing the same concept of a dynamic model and 3D physiologically structured RLM (Lee et al., 2013) possess several problems. First, although the microfluidic liver-on-a-chip system is dynamic, the flow is gravity-induced and cannot be precisely controlled. Additionally, the impact caused by the flow process was not considered when constructing the mathematical model. Second, the diffusion was limited by the PEG gel pillar size and porosity of the encapsulating gel. Third, any substrate and metabolite remaining in the PEG hydrogel were not detected, likely influencing the microsystem. In contrast, our method took the dynamic process into consideration by using the porous and fast-diffusion FAB hydrogel for encapsulating the RLM and by analyzing substrate and metabolite in both the circular medium and the hydrogel. Although the liquid flow problem could not be precisely controlled (Baudoin et al., 2014), a good prediction was obtained for a series of compounds in the absence of the 3D structure provided by the PEG hydrogel.

The applicability of the Bio-PK system can be extended by replacing the metabolic enzymes encapsulated in the FAB hydrogel, circular medium or adding more chambers containing effector or tumor cells for different research purpose. An accurate and reliable *in vitro* model of rat metabolism was provided by Bio-PK metabolic system in terms of both morphology and physiology with applications to modeling the metabolism of other species.
Acknowledgments

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Authorship Contribution

Participated in research design: Ning Zhou, Yuanting Zheng, Weimin Cai, Junfen Xing

Conducted experiments: Ning Zhou, Huiying Yang, Hanmei Chen, Jing Liu, Shanshan Tong, Junfen Xing, Bin Zhu

Contributed new reagents or analytic tools: Ning Zhou, Yuanting Zheng, Xiaoqiang Xiang

Performed data analysis: Ning Zhou, Yuanting Zheng, Xiaoqiang Xiang, Weimin Cai

Wrote or contributed to the writing of the manuscript: Ning Zhou, Yuanting Zheng, Xiaoqiang Xiang, Weimin Cai
References:


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FOOTNOTES

Ning Zhou and Yuanting Zheng contributed equally to the present work. This study was supported by a grant from the National Natural Science Foundation of China [Grant No. 81273584]. The Bio-PK metabolic system presented in this report is the subject of a granted patent by Junfen Xing and Bing Zhu in China (‘In Vitro Analyte Detection for Method and System of Biological Effect’, Ratification NO.ZL200610118351.8).
LEGENDS FOR FIGURES

Fig. 1. (A) Circulation and metabolism in the Bio-PK metabolic system. (B) Sampling process.

Fig. 2. Overview of the Bio-PK metabolic model and the model parameters. \( A \) was for amount [nmol], \( C \) for concentration [\( \mu \text{M} \)], \( V \) for volume [mL], \( D \) for diffusion rate [mL/min] and \( f \) for free fraction. The subscript \( m \) and \( g \) represents for circular medium and FAB hydrogel. \( C_m \) and \( C_g \) means for the concentration of substrate in circular medium and FAB hydrogel, consistent with \( A_m \) and \( A_g \). \( C_u \) defined the substrate concentration that came from hydrogel by circulation. The amount of metabolites was expressed by \( A_{MTB} \). The \( \gamma \) means for the cross-over proportion from the one end of the pipe into the FAB hydrogel. \( Q \) indicated the velocity of flow in peristaltic pump [mL/min]. Assuming that the fluid was plug flow, the time from one end to the other end in the pipeline of the peristaltic pump was \( \tau_1 \) and the time that migrated in the gel was \( \tau_2 \). \( P_{oct} \) the octanol-water-based medium : hydrogel partition.

Fig. 3. Release of RLM from the FAB hydrogel in the Bio-PK metabolic system with flow speed 6.8mL/min, compared with control data in static condition (Yang et al., 2013); \( n = 3 \), mean \( \pm \) S.D.

Fig. 4. Metabolism of the model substrates dextromethorphan (CYP2D2) (A), phenacetin (CYP1A2) (B), chlorzoxazone (CYP2E1) (C), nifedipine (CYP3A1/2) (D) and losartan (CYP2C11) (E) by RLM and RLM encapsulated in FAB hydrogel, incubated at 37 °C for 24 h with continuous shaking in water-bath in centrifuge tubes; \( n = 3 \), mean \( \pm \) S.D.

Fig. 5. Metabolism of probe drugs of rat CYP2D2 (the product is dextrorphan), CYP1A2 (the product is acetaminophen), CYP2E1 (the product is 6-OH chlorzoxazone), CYP3A1/2 (the product is oxidized nifedipine), and CYP2C11 (the product is E-3174) by immersed FAB hydrogel at different times of pre-incubation. Post-incubation, the reaction mixtures were
incubated for a further hour; \( n = 4 \), mean \( \pm \) S.D.

Fig. 6. Stationary state diffusion of tolbutamide from the medium to the FAB hydrogel in the absence of RLM. Depicted are data points measured in the circular medium (blue circles) and the FAB hydrogel (red blocks). The blue line represents the fit to the circular medium data points, whereas the red line represents the fit to the RLM-FAB hydrogel data points; \( n = 3 \), mean \( \pm \) S.D.

Fig. 7. Time course of tolbutamide concentrations in the circular medium at different incubation concentrations. Data fitted for three different incubations 100 \( \mu M \) (green), 150 \( \mu M \) (blue), and 200 \( \mu M \) (red) tolbutamide from experimental data obtained using the Bio-PK Metabolic System; \( n = 4 \), mean \( \pm \) S.D.

Fig. 8. Total amount of 4-hydroxytolbutamide obtained over time in the circular medium and FAB hydrogel at different incubation concentrations. Data fitted for the three different incubations: 100 \( \mu M \) (green), 150 \( \mu M \) (blue), and 200 \( \mu M \) (red) tolbutamide from experimental data obtained using the Bio-PK Metabolic System; \( n = 4 \), mean \( \pm \) S.D.

Fig. 9. Total amount of tolbutamide in the FAB hydrogel at different incubation concentrations. Data fitted for the three different incubations 100 \( \mu M \) (green), 150 \( \mu M \) (blue), and 200 \( \mu M \) (red) tolbutamide from experimental data obtained using the Bio-PK Metabolic System; \( n = 4 \), mean \( \pm \) S.D.

Fig. 10. Extrapolation of the \textit{in vitro} RLM-FAB hydrogel specific intrinsic clearance obtained from the Bio-PK experiment to the \textit{in vivo} data (Sugita et al., 1982) and previous sandwich experiment (Treijtel et al., 2004) by intravenous injection. Data (*) (Sugita et al., 1982) represent the plasma concentration in the rat after i.v. bolus dose (80 mg/kg) after 6 h. Simulated plasma concentrations were obtained using values for the specific intrinsic clearances obtained by fitting
to the Bio-PK metabolic system experimental data for incubations of 100, 150, and 200 μM tolbutamide, in Simcyp software. The dashed line shows the predicted result using the specific intrinsic clearance value from the sandwich culture experiment.

Fig. 11. Extrapolation of the *in vitro* RLM-FAB hydrogel specific intrinsic clearance obtained from the Bio-PK experiment to the *in vivo* data (Sun et al., 2014) by oral administration. Data (*) (Sun et al., 2014) represent the plasma concentration in the rat after oral administration of 1 mg/kg for 48 h. Simulated plasma concentrations using values for the specific intrinsic clearances obtained by fitting the Bio-PK metabolic system experimental data for incubations of 100, 150, and 200 μM tolbutamide, in Simcyp software.
# TABLES

## Table 1. Parameters used in Bio-PK mathematical model.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Units</th>
<th>Initial state/estimated range</th>
<th>Estimated parameter values</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A_m(t) )</td>
<td>nmol</td>
<td>( A_m(0)=A_m;0 )</td>
<td>[ \text{amount of substrate in circular medium} ]</td>
<td></td>
</tr>
<tr>
<td>( A_g(t) )</td>
<td>nmol</td>
<td>( A_g(0)=0 )</td>
<td>[ \text{amount of substrate in RLM-FAB hydrogel} ]</td>
<td></td>
</tr>
<tr>
<td>( A_{mt}(t) )</td>
<td>nmol</td>
<td>( A_{mt}(0)=0 )</td>
<td>[ \text{amount of metabolites} ]</td>
<td></td>
</tr>
<tr>
<td>( C_m(t) )</td>
<td>( \mu M )</td>
<td>( C_m(0)=C_m;0 )</td>
<td>[ \text{concentration of substrate in circular medium} ]</td>
<td></td>
</tr>
<tr>
<td>( C_g(t) )</td>
<td>( \mu M )</td>
<td>( C_g(0)=0 )</td>
<td>[ \text{concentration of substrate in FAB hydrogel} ]</td>
<td></td>
</tr>
<tr>
<td>( C_m(t-\tau_1) )</td>
<td>( \mu M )</td>
<td>( C_m(0-\tau_1)=C_m(0) )</td>
<td>[ \text{liquid concentration into the pipe before the ( \tau_1 ) moment} ]</td>
<td></td>
</tr>
<tr>
<td>( f_g )</td>
<td>-</td>
<td>( 0&lt;f_g&lt;1 )</td>
<td>[ 0.905\pm0.110 ]</td>
<td>[ \text{free fraction from FAB hydrogel to circular medium} ]</td>
</tr>
<tr>
<td>( D )</td>
<td>mL/min</td>
<td>( 0.036 )</td>
<td>[ 0.036 ]</td>
<td>[ \text{molecular diffusion coefficients of tolbutamide obtained by experiments} ]</td>
</tr>
<tr>
<td>( CL_g )</td>
<td>mL/min</td>
<td>-</td>
<td>[ 0.0109\pm0.00107 ]</td>
<td>[ \text{in vitro RLM-FAB hydrogel specific intrinsic clearance} ]</td>
</tr>
<tr>
<td>( \tau_2 )</td>
<td>s</td>
<td>-</td>
<td>[ 4.40\pm0.291 ]</td>
<td>[ \text{the time that flew through the gel} ]</td>
</tr>
<tr>
<td>( C_u(t) )</td>
<td>( \mu M )</td>
<td>-</td>
<td>-</td>
<td>[ \text{substrate concentration that came from hydrogel by circulation} ]</td>
</tr>
<tr>
<td>( \gamma )</td>
<td>-</td>
<td>( 0&lt;\gamma&lt;1 )</td>
<td>[ 0.470\pm0.073 ]</td>
<td>[ \text{cross-over proportion from the one end of the pipe into FAB hydrogel} ]</td>
</tr>
<tr>
<td>( Q )</td>
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<td>6.88</td>
<td>[ \text{the velocity of flow in peristaltic pump} ]</td>
</tr>
<tr>
<td>( \tau_1 )</td>
<td>s</td>
<td>9.2</td>
<td>9.2</td>
<td>[ \text{time from one end to the other end in the pipeline of the peristaltic pump} ]</td>
</tr>
<tr>
<td>( V_m )</td>
<td>mL</td>
<td>3.6</td>
<td>3.6</td>
<td>[ \text{volume of circular medium} ]</td>
</tr>
<tr>
<td>( V_g )</td>
<td>mL</td>
<td>0.3025</td>
<td>0.3025</td>
<td>[ \text{volume of RLM-FAB hydrogel} ]</td>
</tr>
<tr>
<td>( f_{aw} )</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>[ \text{octanol-water-based medium : hydrogel partition} ]</td>
</tr>
<tr>
<td>( f_{am} )</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>[ \text{free fraction from circular medium to FAB hydrogel} ]</td>
</tr>
</tbody>
</table>

**Notes:**
- This article has not been copyedited and formatted. The final version may differ from this version.
a. \text{Amount (nmol)} = \text{coefficient (mL/min)} \times \text{concentration (μmol/L)}; \text{Coefficient (mL/min) can be } Q, CL_{up}, D.

b. Before the experiment began, peristaltic pump was started to fill the recirculating pipeline with circular medium. So, \text{C}_m(t) = \text{C}_m(0)
Figure 1

A. Diagram showing a peristaltic pump connected to a reaction chamber containing RLM-FAB hydrogel in a circular medium at 37°C.

B. Illustration depicting RLM-FAB hydrogel and a circular medium.
Figure 3

Concentration of released microsomes (mg/mL) vs. time (h)

- Control
- 0.3%-8.5% FAB hydrogel in Bio-PK system
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

Graph showing the amount of 4-hydroxytolbutamide (nmol) over time (min) for different concentrations of TOL: 100 µM (green triangles), 150 µM (blue squares), and 200 µM (red circles). The data points are connected by linear lines, indicating a direct proportional relationship between time and amount of 4-hydroxytolbutamide.
Figure 11
APPLICATION OF A NEW DYNAMIC MODEL TO PREDICT THE IN VITRO INTRINSIC CLEARANCE OF TOLBUTAMIDE USING RAT MICROSOMES ENCAPSULATED IN A FAB HYDROGEL

Ning Zhou, Yuanting Zheng, Junfen Xing, Huiying Yang, Hanmei Chen, Xiaoqiang Xiang, Jing Liu, Shanshan Tong, Bing Zhu, Weimin Cai

Drug Metabolism and Disposition

Supplemental data:

In order to compare the traditional RLM (a static model) to the Bio-PK model (a dynamic model), two figures were added as Supplemental data.

We obtained values for the specific intrinsic liver clearance of 4.06, 3.21, and 3.53 μL/min/mg for the incubations of 100, 150, and 200 μM tolbutamide, respectively, which were 3.6 μL/min/mg in average and comparatively smaller than the CL_int of 6.2 μL/min/mg obtained previously (Ashforth et al., 1995a). At the same time, we also use the traditional RLM (a static model) as parallel control design and the result of CL_int was 1.6 μL/min/mg. The figures (Supplemental Figure 1 and Supplemental Figure 2) below were predicted in vivo results by applying the three data (1.6 μL/min/mg for “RLM control” obtained by parallel experiment (a static model), 3.6 μL/min/mg for the average value of “Bio-PK system” and 6.2 μL/min/mg for “Reference value” (a static model) (Ashforth et al., 1995a)) in Simcyp software.
The predicted *in vivo* clearance of tolbutamide was 0.059, 0.096, and 0.153 L/h/kg for intravenous injection and 0.047, 0.105, and 0.180 L/h/kg for oral administration, respectively to RLM control, Bio-PK system and Reference value. Compared with the reported *in vivo* clearance of 0.09 L/h/kg (i.v.) and 0.10 L/h/kg (oral), only the fold error of RLM control for oral administration was above 2.

However, the predictions of Bio-PK system were more accurate for both drug administrations.

Supplemental Figure 1 and Supplemental Figure 2 showed the results together with those from the model calculations using the average specific intrinsic clearance value from the plasma data obtained for both intravenous injection and oral administration. It was evident from the data that they are all in good agreement. Data from 2 static models of “traditional” RLM incubation was also included for comparison purposes. Thus the *in vivo* prediction process could be compared directly. The comparison indicated that the results from the Bio-PK system were more similar to the *in vivo* data compared with the results obtained for the static models both from parallel control experiments and reference data (Ashforth et al., 1995a). This result confirmed the practical interest of simulating *in vivo* metabolism data using the Bio-PK metabolic system by incorporating dynamic flow and 3D structure of RLM.

Prediction was improved by incorporating of dynamic flow and 3D structure in Bio-PK metabolic system.
Supplemental Figure 1. Extrapolation of the *in vitro* intrinsic clearance obtained from the RLM control experiment, Bio-PK experiment and reference data (Ashforth et al., 1995a) to the *in vivo* data (Sugita et al., 1982) by intravenous injection. Data (*) (Sugita et al., 1982) represent the plasma concentration in the rat after *i.v.* bolus dose (80 mg/kg) after 6 h. Simulated plasma concentrations were obtained using values for the intrinsic clearances obtained from the RLM control experiment, Bio-PK experiment and reference data, in Simcyp software.

Supplemental Figure 2. Extrapolation of the *in vitro* intrinsic clearance obtained from the RLM control experiment, Bio-PK experiment and reference data (Ashforth et al., 1995a) to the *in vivo* data (Sun et al., 2014) by oral administration. Data (*) (Sun et al., 2014) represent the plasma concentration in the
rat after oral administration of 1 mg/kg for 48 h. Simulated plasma concentrations were obtained using values for the intrinsic clearances obtained from the RLM control experiment, Bio-PK experiment and reference data, in Simcyp software.
References

