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Article

Novel *in vitro* dynamic metabolic system for predicting the human pharmacokinetics of tolbutamide

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Abstract

Liver metabolism is commonly considered the major determinant in drug discovery and development. Many *in vitro* drug metabolic studies have been developed and applied to understand biotransformation. However, these methods have disadvantages, resulting in inconsistencies between *in vivo* and *in vitro* experiments. A major factor is that they are static systems that do not consider the transport process in the liver. Here we developed an *in vitro* dynamic metabolic system (Bio-PK metabolic system) to mimic the human pharmacokinetics of tolbutamide. Human liver microsomes (HLMs) encapsulated in a F127'-Acr-Bis hydrogel (FAB hydrogel) were placed in the incubation system. A microdialysis sampling technique was used to monitor the metabolic behavior of tolbutamide in hydrogels. The measured results in the system were used to fit the *in vitro* intrinsic clearance of tolbutamide with a mathematical model. Then, a PBPK model that integrated the corresponding *in vitro* intrinsic clearance was developed to verify the system. Compared to the traditional incubation method, reasonable PK profiles and the *in vivo* clearance of tolbutamide could be predicted by integrating the intrinsic clearance of tolbutamide obtained from the Bio-PK metabolic system into the PBPK model. The predicted maximum concentration (C_{max}), area under the concentration-time curve (AUC), time to reach the maximum plasma concentration (T_{max}) and *in vivo* clearance were consistent with the clinically observed data. This novel *in vitro* dynamic metabolic system can compensate for some limitations of traditional incubation methods; it may provide a new method for screening compounds and predicting pharmacokinetics in the early stages, supporting the development of compounds.

Keywords: physiologically based pharmacokinetic model; FAB hydrogel; microdialysis; tolbutamide; Bio-PK metabolic system; dynamic; human liver microsomes

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Introduction

In vitro studies of drugs are usually prioritized over *in vivo* ones, especially in humans. In the early stages of drug discovery and development, *in vitro* data can be used not only to rapidly screen compounds but also to understand the mechanisms and guide the further study of drugs and decision-making[1, 2].

The liver plays an important role in the human body, involving the metabolism and elimination of drugs[3, 4]. Metabolites may have more activity or toxicity than the parent drug, which impacts drug efficacy and safety in humans[5-7]. Thus, biotransformation in the liver is a determinant in the overall disposition of drugs. A reliable *in vitro* metabolic system to predict metabolic behavior in the human body is highly desired.

Currently, many *in vitro* metabolic systems have been developed to explain hepatic extraction. Kinetic parameters, such as V_{max} and K_m, can be determined *in vitro* using hepatocytes, microsomes, cytoplasm, the S-9 fraction or liver slices extracted from the liver of humans and other laboratory animals[8-11]. The intrinsic clearance ($CL_{int}=V_{max}/K_m$ under linear conditions) obtained from these models can be converted to *in vivo* intrinsic clearance using various scaling methods, based on the amount of enzyme present *in vivo*[12]. However, these systems have several limitations, such as a scarce and inconsistent metabolic rate. As the gold standard, hepatocytes are prone to inactivation over time in culture. Moreover, available primary human hepatocytes are fewer in number and scarce, as they are mainly derived from poor-quality or poorly transplanted substances[13-15]. The incubation time for microsomes

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and recombinases is often short, which limits the generation of sufficient metabolic turnover and affects prediction of the clearance rate[16-18]. More importantly, the systems described above are static, simplifying the circulation processes occurring in the liver[19]. To better understand the metabolic behavior, a new *in vitro* metabolic system must be developed.

In our previous study, a hydrogel carrier (F127-acrylamide-bisacrylamide, FAB hydrogel) with a polymer thermosensitive material was invented. FAB hydrogel is a reverse-phase hydrogel, with the appropriate proportion of hydrogel presenting a loose, porous three-dimensional structure suitable for liver microsomal loading. It has been confirmed that microsomes encapsulated in hydrogel still have good metabolic activity[20]. Compared to ordinary microsomes, microsomal hydrogels can be used for long-term incubation reactions due to their ability to maintain longer metabolic activity and metabolic turnover[20]. We have further developed an *in vitro* dynamic metabolic cycle system (Bio-PK system) to better mimic and understand the metabolic behavior. Rat liver microsomes encapsulated in a FAB hydrogel were combined with the Bio-PK system, which successfully predicted the *in vivo* metabolism of tolbutamide[21]. A dynamic bio-pharmacokinetic/pharmacodynamic (PK/PD) system was established and assessed to predict the PK parameters and PD effects of the model drug cyclophosphamide[22]. However, drug research in humans is the ultimate goal of drug development. Due to differences in species, it is desirable to estimate clearance in humans. Based on the above results, it is important to further apply the Bio-PK system combined with microsomal hydrogel to predict human metabolism.

The objectives of the present work are 1) to prepare a microsomal hydrogel that can encapsulate human liver microsomes; 2) to construct the Bio-PK system to obtain the *in vitro* intrinsic clearance of tolbutamide; 3) to build the PBPK model via integration with the *in vitro* intrinsic clearance obtained from the Bio-PK system to predict the clinically pharmacokinetics of tolbutamide; and, ultimately, 4) to evaluate the potential and reliability of this system in predicting human metabolic behavior and to support the further development of the system.

Materials and methods

Chemicals and reagents

Pluronic F-127, tolbutamide, and 4-hydroxytolbutamide were purchased from Sigma Aldrich (St Louis, MO, USA). The BCA Protein Assay Kit, 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). Phenacetin was acquired from Aladdin (Shanghai, China). Mixed gender pooled human liver microsomes (prepared from at least 20 donors) were obtained from Corning Life Sciences (Tewksbury, MA, USA), and β-Nicotinamide adenine dinucleotide 2′-phosphate (NADPH) was acquired from Roche (Mannheim, Germany).

HPLC-grade methanol was purchased from TEDIA Company, Inc (Fairfield, USA). Water was purified using a Milli-Q water system (Bedford, MA, USA). The distilled water was used for the extraction and preparation of samples. Other reagents and chemicals were of analytical grade.

Ringer's solution (145 mmol/L Na^+, 1.2 mmol/L Ca^{2+}, 4 mmol/L K^+, and 0.1 mmol/L ascorbic acid, pH 7.0) and phosphate buffer saline (PBS) solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L sodium phosphate dibasic, and 2 mmol/L potassium phosphate monobasic, pH 7.4) were prepared in our laboratory.

Synthesis of F-127′ and preparation of HLM-encapsulated FAB hydrogel

The synthesis of F-127′ and the FAB hydrogel preparation were as previously described[20]. Briefly, F-127′, a triblock copolymer, poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO99-PPO65-PEO99), was derived at both ends of the F127 polymer using acryloyl chloride to generate double bonds. The FAB hydrogel system was prepared using Acr-Bis solution, F127′, APS, TEMED and PBS.

The hydrogel still had a thermo-sensitive property such that the polymer chain presented a diastolic state at 4 °C and a systolic state at 37 °C. The prepared FAB hydrogel was immersed in an HLM solution for 48 h at 4 °C before metabolic tests.

Liquid chromatography–tandem mass spectrometry method

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was conducted using an Agilent 1200 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an API 4000 Q Trap mass spectrometer (AB Sciex, Darmstadt, Germany) equipped with an API electrospray ionization (ESI) source.

The chromatography separation was performed on an Agilent Eclipse XDB-C18 column (150 mm×2.1 mm id, 5 μm; Agilent Technologies, Waldbronn, Germany). The column temperature was maintained at 30 °C. The mobile phase consisted of (A) methanol + 0.2% acetic acid and (B) water + 0.2% acetic acid by an isocratic elution of 60% A at 0–5 min. The flow rate was 0.3 mL/min, and the injection volume was 5 μL.

The ESI source was operated in negative ionization mode with an ion spray voltage at 4.5 kV. The source temperature was set to 500 °C. Multiple reaction monitoring (MRM) was performed for each sample. The ion transitions were mass-to-charge ratios of (*m/z*) 269.1 to 169.8 for tolbutamide, 285.0 to 185.8 for 4-hydroxytolbutamide and 178.1 to 148.8 for phenacetin, which was used as the internal standard. All data were analyzed using Analyst software, version 1.6.

Construction of the Bio-PK metabolic system

The Bio-PK metabolic system consisted of a peristaltic pump and an incubation system, tightly connected by a recirculation pipeline. HLMs encapsulated in the FAB hydrogel were placed in the incubation system. Substrate and NADPH were also added to the incubation system. The final incubation system was placed in a thermostatic metal bath. A microdialysis sampling technique was used to obtain the free concentration levels of drugs in hydrogels. A schematic of the Bio-PK system is shown in Figure 1.

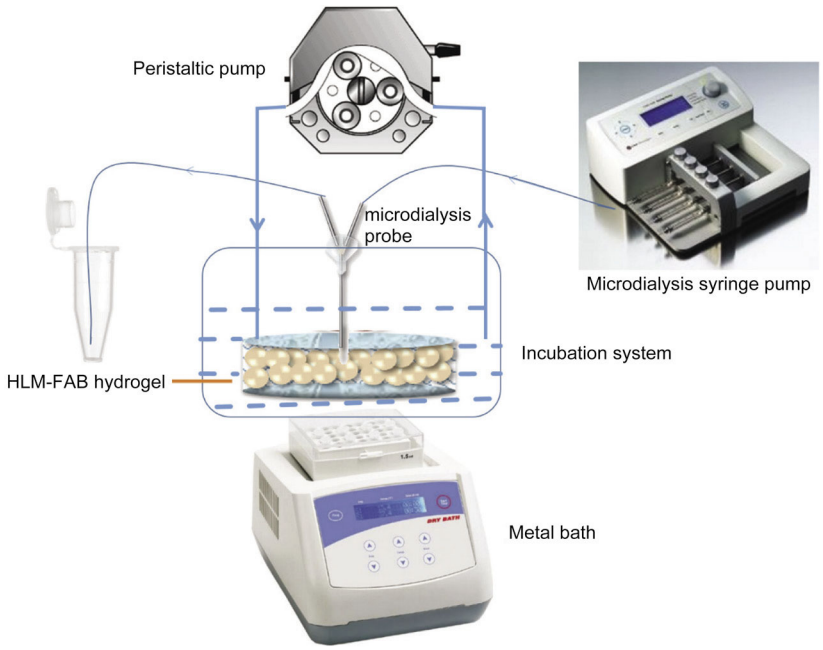

Figure 1. Schematic of the Bio-PK system, consisting of a peristaltic pump, incubation systems and microdialysis systems. HLMs encapsulated in the FAB hydrogel were placed in the incubation system, which was placed in a thermostatic metal bath. The microdialysis system consisted of a microdialysis syringe pump and microdialysis probes.

Microdialysis sampling

Microdialysis sampling techniques were integrated into the Bio-PK system to better obtain samples from the FAB hydrogels in real time. The microdialysis systems consisted of a microdialysis syringe pump (CMA/400, CMA, Stockholm, Sweden) and microdialysis probes. The dialysis probes (2 mm in length) have a molecular weight cut-off of 6 kDa (Microbiotech/se AB, Stockholm, Sweden). The substance used as the membrane material was PES (polyether sulphone). The microdialysis probe was inserted into the FAB hydrogel. Thereafter, the probes were perfused with Ringer's solution at a flow rate of 1 μL/min, using the CMA microdialysis syringe pump for delivery of the perfusion solution. The perfusate was passed through a 0.22-μm nylon filter before use. The perfusion fluid entered the probe through the inlet tubing at a flow rate of 1 μL/min, passed the membrane and was then transported through the outlet tubing, collected in a microtube and analyzed by LC-MS/MS.

Recovery assessment of microdialysis probes

The recovery for the microdialysis probe was determined using the no net flux method (NNF)[23-25]. The microdialysis probe was inserted into the FAB hydrogel from the incubation system of the Bio-PK system at 37 °C containing tolbutamide (200 μmol/L, 100 μmol/L and 50 μmol/L) and was perfused at 1 μL/min with Ringer's solution containing different concentrations of tolbutamide (5, 20, 80, 150, 250 and 300 μmol/L, Cp). The dialysate samples (Cd) were collected continuously every 15 minutes (n=3) for each concentration of perfusion solution and were analyzed by LC-MS/MS. Linear regression analysis was performed with the perfusion solution (Cp) as the abscissa and the concentration difference between the microdialysate samples and the perfusion solution (Cd−Cp) as the ordinate. The slope was the relative recovery (RR) of the probe, and the intercept of the straight line and the X axis was the free drug concentration (Cf).

The recovery of 4-hydroxytolbutamide was obtained in a similar manner as the determination of tolbutamide recovery. The concentration of 4-hydroxytolbutamide in the vial of the dialysis medium was 10 μmol/L, and six perfusates contained different concentrations of 4-hydroxytolbutamide (1, 10, 20, 40, 80 and 100 μmol/L).

Diffusion coefficients

To determine the diffusion coefficients, the FAB hydrogel containing microsomes with a final concentration of 0.5 mg/mL was placed in the incubation system. The microdialysis probe was inserted into the FAB hydrogel. Thereafter, the probes were perfused with Ringer's solution at a flow rate of 1 μL/min. Following the addition of 200 μmol/L tolbutamide, the dialysate samples were collected at 5, 10, 25, 40, 55, 70, 85, 100 and 120 min and were analyzed by LC-MS/MS.

Flow rate for the Bio-PK metabolic system

To determine the final flow rate of the Bio-PK metabolic system, different flow rates were set to examine the release of microsomes from the FAB hydrogel and the production of metabolites.

HLMs encapsulated in the FAB hydrogel (at a final concentration of 0.5 mg/mL) were placed in the incubation system at 37 °C, and the total circulating fluid volume was 3 mL. The system was circulated for 24 h with a flow rate of 0 mL/min,

21 mL/min, 42 mL/min, 63 mL/min or 85 mL/min, and 50 μL of circulating fluid was collected at 0.5, 1, 2, 4, 6, 8 and 24 h for protein quantitation using the BCA Protein Assay Kit.

To further confirm the flow rate, 200 μmol/L tolbutamide and 1 mmol/L NADPH were added to the same incubation system above to detect metabolites with a flow rate of 5 mL/min, 10 mL/min, 20 mL/min or 40 mL/min.

Metabolism of tolbutamide in the Bio-PK metabolic system

Following the above description to construct the Bio-PK metabolic system, the volume and flow rate of the total circulating fluid were 3 mL and 10 mL/min, respectively. FAB hydrogel was immersed in HLMs for 48 h at 4 °C and was immediately mixed with PBS buffer containing tolbutamide and NADPH (1 mmol/L) to a final protein concentration of 0.5 mg/mL. The incubation system was preincubated to 37 °C for 5 min in a thermostatic metal bath. The cyclic reactions were initiated with the addition of tolbutamide stock solutions (50, 100 and 200 μmol/L final concentrations). Samples were gently mixed and maintained at 37 °C for 4 h. Samples of FAB hydrogel and circulating fluid were taken separately after incubations of 0, 20, 40, 60, 80, 100, 120, 180, and 240 min. The samples of circulating fluid were immediately added with 2 times methanol containing the internal standard (IS; phenacetin) and mixed for 2 min on a vortex mixer to terminate the reaction. The solution was centrifuged at 138 000×*g* for 10 min. The supernatant was transferred to a separate container for LC-MS/MS analysis. The dialysate samples from the FAB hydrogel were directly transferred to microtubes and analyzed by LC-MS/MS within 48 h.

Mathematical model

Based on the circulating perfusion and mass balance of the Bio-PK system, a mathematical model was developed to describe the above dynamic process and was fitted to the clearance (CL_g) in FAB hydrogel using MATLAB (MathWorks, Inc, Natick, MA, USA). The model considers factors such as diffusion in the FAB hydrogels, the interchange between the FAB hydrogels and the circulating fluid, HLM protein binding and dynamic circulation. The *in vitro* intrinsic clearance (CL_{int}) in human is based on the assumption that HLMs per mass unit have the same metabolic capacity. A scheme of the mathematical model is shown in Figure 2. The relevant formula is as follows:

$$\frac{dC_m(t)V_m}{dt} = D(C_g(t) - f_m C_m(t)) + (1-\gamma)QC_m\,(t-\tau_1) - \gamma QC_u(t) - QC_m(t)$$

$$\frac{dC_g(t)V_g}{dt} = -D(C_g(t) - f_m C_m(t)) + \gamma QC_m(t-\tau_1) - CL_g C_g(t) - \gamma QC_u\,(t-\tau_2)$$

$$C_u(t) = C_g(t+\tau_2) - CL_g C_g(t+\tau_2)\tau_2 / V_g$$

$$\frac{A_{MTB}(t)}{dt} = CL_g C_g(t)$$

where C_m and C_g are the concentration and V_m and V_g the volume of substrate in the circular medium and FAB hydrogel, respectively; C_u is the substrate concentration that came from the hydrogel due to circulation; and the amount of metabolites is expressed by AMTB. Q is the flow rate of the peristaltic pump; D is the diffusion coefficient; f_m is the free fraction; γ is the crossover proportion from one end of the pipe into the FAB hydrogel; τ_1 is the time from one end to the other in the pipeline of the peristaltic pump; and τ_2 is the migration time in the gel.

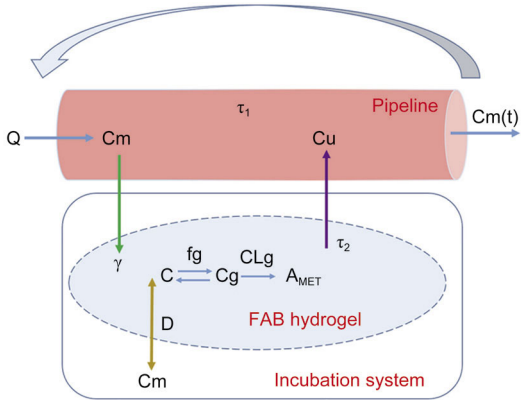

Figure 2. Dynamic disposition scheme of the Bio-PK system. C_m, concentration of substrate in circular medium; C_g, concentration of substrate in FAB hydrogel; C_u, concentration of substrate flow out of FAB hydrogel; A_{MTB}, amount of metabolites; Q, flow rate; D, diffusion coefficients; f_g, free fraction; γ, proportion flowing into FAB hydrogel; τ_1, time flowing through pipeline; τ_2, time flowing through FAB hydrogel; CL_g, clearance in FAB hydrogel.

PBPK model development

To better verify the Bio-PK system, a PBPK model was developed to predict the PK profile and CL of tolbutamide using the Simcyp™ population-based ADME simulator (V15; Simcyp Limited, Sheffield, UK). The PBPK model was simulated by integrating the in silico and *in vitro* data of the drug in healthy volunteer population databases from the Simcyp simulator. The Monte Carlo method allows the random generation of a unique set of physiological parameters for each virtual subject, such as anatomic, demographic, and tissue-specific parameters. The variation in each variable was generated by random sampling of each variable within its defined distribution for each simulated virtual subject. The default trial designed by Simcyp was selected to build the model for tolbutamide. A virtual population of 100 healthy volunteers (10 trials with 10 subjects each), aged 20–50 years with a female/male ratio of 0.5, was used to simulate PK following single oral doses of 500 mg of tolbutamide.

A minimal PBPK distribution model with tissue partition coefficients was predicted by the Rodgers method[26], and a first-order absorption model was used to describe the distribution and absorption. The elimination process was characterized by the intrinsic clearance of CYP2C9 and by kidney clearance.

The accuracy of the PBPK model was assessed by compar-

Table 1. *In vitro* microdialysate recoveries for tolbutamide and 4-hydroxytolbutamide.

Concentration (μmol/L)	Recovery (%)
Tolbutamide	
200	21.88±1.16
100	26.50±1.02
50	22.83±0.68
Average	23.74±1.35
4-Hydroxytolbutamide	
8	33.46±0.16

Data are expressed as mean±standard deviation (n=3).

ing the ratio between the predicted and observed pharmacokinetic parameters (fold error), together with visual inspection. If observed>predicted, the fold error=observed/predicted; otherwise, fold error=predicted/observed. A model with a fold error of less than two is generally considered a precise model[27-31].

Results

In vitro relative recovery of probes

The *in vitro* recoveries of probes for tolbutamide and 4-hydroxytolbutamide are shown in Table 1. The actual concentration of tolbutamide and 4-hydroxytolbutamide in the FAB hydrogel could be corrected by an average recovery of 23.74% and 33.46%, respectively.

Diffusion coefficients

The diffusion coefficients of tolbutamide from the medium into FAB hydrogel are shown in Figure 3. A final concentration of 0.5 mg/mL microsomes was encapsulated in 300 μL of FAB hydrogel. Following the addition of drug, tolbutamide is rapidly diffused into the FAB hydrogel due to the leakage effect between the hydrogel and the medium; equilibrium was reached after 70 min as the reaction progressed. Therefore, the diffusion coefficient of tolbutamide was estimated as 0.004 mL/min.

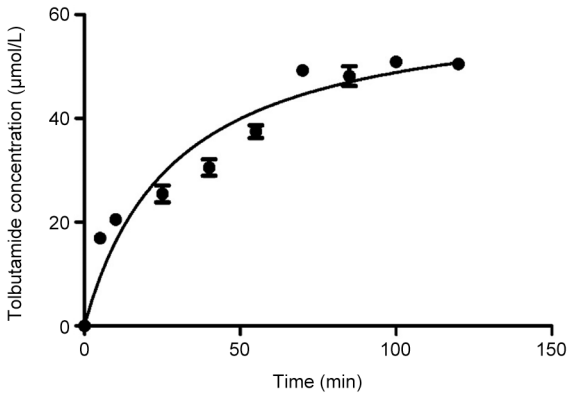

Figure 3. Diffusion of tolbutamide from medium to FAB hydrogel; n=3, mean±SD.

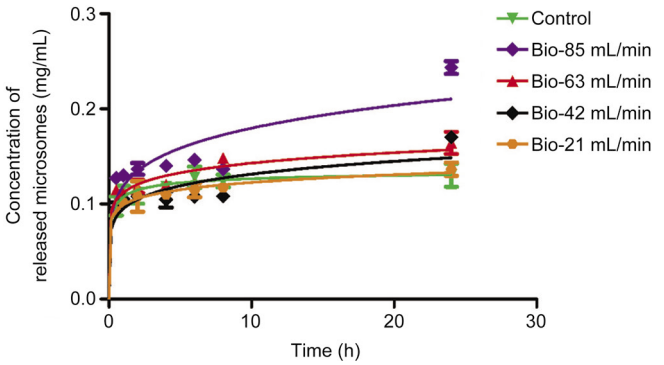

Figure 4. Release of HLM protein from FAB hydrogel in the Bio-PK metabolic system under various flow rates, compared to the control group under static conditions; n=3, mean±SD.

Flow rate for the Bio-PK metabolic system

The release of microsomal protein from the FAB hydrogel was determined under different flow rates, as shown in Figure 4. Data in the control group indicate that the release of microsomal protein from the FAB hydrogel is in a static state.

With the increase in flow rate, the release of microsomal protein from the FAB hydrogel showed a tendency to increase, indicating that it was gradually washed out of the hydrogel. When the flow rate was 21 mL/min, the release of microsomes was consistent with that of the control group. The concentration of microsomal protein increased slightly in the first few minutes and then immediately reached the plateau stage, indicating that the microsomal proteins are firmly locked inside the FAB hydrogel and remain stable for a long period. However, when the flow rate was above 21 mL/min, the release of microsomes was higher than in the control group. With increasing flow rate, the time at which the microsomes washed out of the hydrogel gradually advanced. The microsomes washed out of the hydrogel after approximately 12 h at 42 mL/min. At a flow rate of 63 mL/min, the time advanced to 6 h, and when the flow rate increased to 85 mL/min, the microsomes were rinsed out first.

Based on the above results, we tested four flow rates to further examine the metabolic status of tolbutamide as the flow

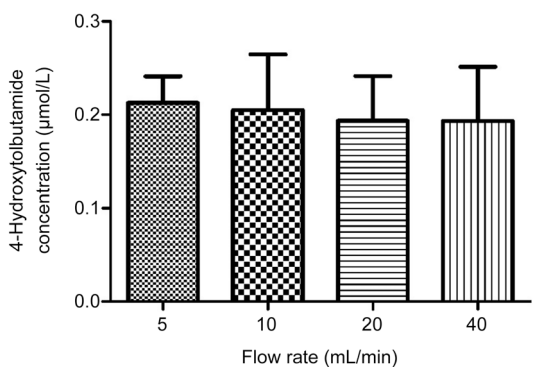

Figure 5. Metabolic status of tolbutamide at various flow rates. n=3, mean±SD.

rate varied. The data are shown in Figure 5. In the figure, the metabolites of tolbutamide formed are basically the same at different flow rates, consistent with our expectation. This indicates that the microsomes are well encapsulated by the FAB hydrogel at a flow rate of less than 42 mL/min. Considering the destruction of the hydrogel and the long-term decrease in loading capacity at high flow rates, a flow rate of 10 mL/min was selected for the Bio-PK system.

Tolbutamide metabolism in the Bio-PK metabolic system

After successful construction of the Bio-PK metabolic system, various concentrations of tolbutamide were placed in the system for circulating incubation. The related results are shown in Figures 6, 7 and 8. In the Bio-PK system, the production of 4-hydroxytolbutamide consistently increased over time and was certainly correlated with the concentration of the substrate. As the reaction progressed, the amount of tolbutamide in the FAB hydrogel gradually increased and finally reached the saturation level. The volume of the FAB hydrogel was small, resulting in less impact on the substrate concentration in the circulating fluid after the substrate entered the FAB hydrogel over time. Therefore, the concentration of tolbutamide in the circulating fluid was essentially unchanged. The

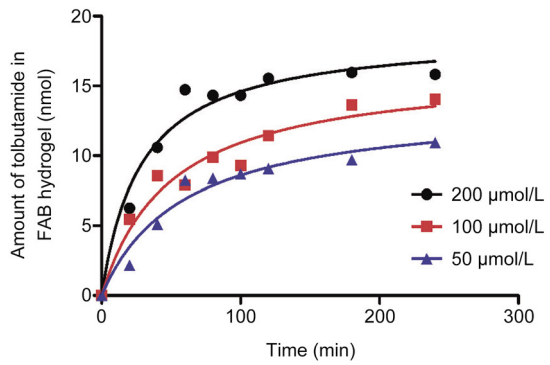

Figure 6. Different concentrations of tolbutamide over time in the FAB hydrogel obtained using the Bio-PK metabolic system; *n*=3, mean±SD.

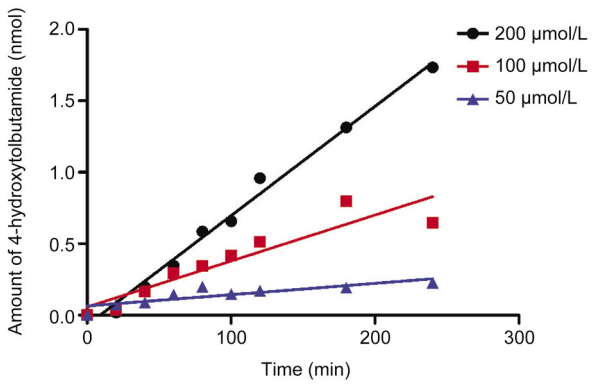

Figure 7. Amount of 4-hydroxytolbutamide formed by different concentrations of tolbutamide over time obtained using the Bio-PK metabolic system; *n*=3, mean±SD.

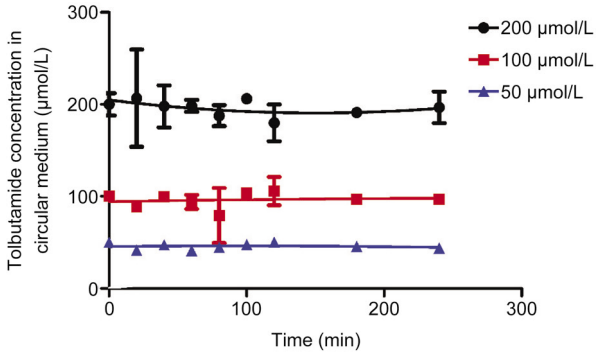

Figure 8. Concentration of tolbutamide over time in medium obtained using the Bio-PK metabolic system; *n*=3, mean±SD.

above data will be applied to the mathematical model to fit tolbutamide clearance.

Mathematical model

To better describe the convection-diffusion-reaction characteristics inside the Bio-PK system, a mathematical model was developed using MATLAB by optimizing the discrete model of the parameter values, considering the experimental data and assuming a relative error model. In this model, CL_g is the intrinsic substrate clearance in the FAB hydrogel. Based on the metabolism of tolbutamide in the Bio-PK metabolic system, CL_g was fitted to 0.0102 mL/min for 200 μmol/L, 0.009 mL/min for 100 μmol/L, and 0.0072 mL/min for 50 μmol/L. Thus, the corresponding intrinsic clearance of tolbutamide was 6.8 (200 μmol/L), 6.0 (100 μmol/L) and 4.8 (50 μmol/L) μL/min/mg, respectively.

PBPK model for tolbutamide

The PBPK model of tolbutamide was built based on the parameters in Table 2. The simulated PK profiles after oral doses of 500 mg of tolbutamide are shown in Figure 9. Compared to the intrinsic clearance obtained from the previous traditional incubation method, a good match for the PK profile can be predicted by integrating the intrinsic clearance of tolbutamide obtained from the Bio-PK system into the PBPK model. The simulated C_{max}, AUC and T_{max} of tolbutamide, based on the average intrinsic clearance (5.87 μL/min/mg), were 36331 ng/mL, 519080 ng·h/mL, and 3.6 h, respectively. All were within 2-fold error of the observed results (C_{max}=33500 ng/mL, AUC=483223 ng·h/mL and T_{max}=4.1 h)[32] (Table 3). The literature and predicted *in vivo* CL values for tolbutamide are shown in Table 4. The predicted *in vivo* CL values for tolbutamide were 1.26 L/h for a 200 μmol/L incubation, 1.11 L/h for a 100 μmol/L incubation and 0.89 L/h for a 50 μmol/L incubation. The mean *in vivo* CL of tolbutamide predicted by the PBPK model using the clearance obtained from the Bio-PK system was 1.09 L/h, in good agreement with (< 2-fold error) the observed results (mean CL=0.84 L/h)[33-38]. However, the predicted mean CL obtained from traditional incubation was 0.38 L/h[39, 40], indicating that the Bio-PK system can better predict metabolic behavior *in vivo* and obtain more accurate *in*

Table 2. Parameters for tolbutamide used in PBPK modeling.

Parameter	Tolbutamide	
	Value	References/Comments
Mol weight (g/mol)	270.3	Drug bank
Log $P_{o:w}$	2.34	Drug bank
pKa	5.16	Drug bank
B/P	0.55	[55]
$f_{u,p}$	0.054	[56]
f_a	0.93	[34]
k_a (h^{-1})	0.52	[34]
Q_{Gut} (L/h)	5.73	Predicted
Permeability	0.95	Predicted
PSA (Å^2)	83.65	Pubchem
HBD	2	Pubchem
V_{ss} (L/kg)	0.105	Predicted with Rogers method
CL_R	0.0019	[54]
Enzyme	CYP2C9	Metabolite: 4-hydroxytolbutamide
CL_{int} (μL/min per milligram)	1.87, 2.28, 6.80, 6.00, 4.80 (mean=5.87)	[39] [40] Bio-PK system
$f_{u,mic}$	0.97	[57]

B/P, blood-to-plasma ratio; $f_{u,p}$, free fraction in plasma; f_a, fraction of dose absorbed; k_a, first-order absorption rate constant; V_{ss}, steady-state volume of distribution; $f_{u,mic}$, free fraction in liver microsome; CL_{int}, intrinsic clearance; CL_R, renal clearance; Q_{Gut}, flow rate for overall delivery of drug to the gut; PSA, polar surface area; HBD, hydrogen bond donors.

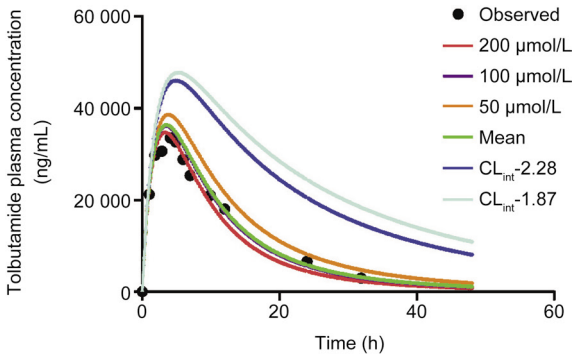

Figure 9. Predicted and observed mean plasma concentration-time profiles of tolbutamide after a single oral dose of 500 mg of tolbutamide. The solid lines represent the mean plasma concentrations predicted by integrating various *in vitro* intrinsic clearances; red, purple, orange and green lines represent the plasma concentration simulated by integration of the intrinsic clearance fitted using the Bio-PK system, whereas blue and pale blue lines represent the integration of the intrinsic clearance obtained from the traditional incubation method[39, 40]. Symbols represent mean observed data[32].

vitro data.

Discussion

Liver metabolism is commonly considered the major determinant in drug discovery and development. Many *in vitro* drug metabolic studies have already been developed and applied to understand biotransformation. These provide some guidance for the screening and development of drugs, as well as the elucidation of metabolic mechanisms and pathways[9]. However, these methods have disadvantages, resulting in inconsistencies between *in vivo* and *in vitro* experiments. A major factor is that they are static systems that do not consider the transport process in the liver.

Based on our previous studies, a dynamic Bio-PK metabolic system that consists of a peristaltic pump and an incubation system containing a rat microsome-encapsulated FAB hydrogel is highly expected to be a new tool for *in vitro* metabolic study[21, 22]. However, the ultimate goal of drug research is focused on human studies. It is often desirable to estimate clearance in humans from measurements performed *in vitro* or in other species. A specific probe substrate is more suitable and can more clearly illustrate the dynamic system. Tolbutamide—a classical probe substrate for CYP2C9 based on a representative list of preferred and acceptable *in vitro* probe substrates recommended by FDA guidance—was chosen as the model drug. Many *in vitro* and *in vivo* studies have enabled comparison and validation of the dynamic system. In addition, it has been reported that the drug has a low extraction rate[41, 42], and most current incubation times are short (approximately 30 min), which affects tolbutamide metabolism. The HLMs encapsulated in the FAB hydrogel can better extend microsomal activity at long incubation times. In the dynamic system, tolbutamide quickly diffused in the hydrogel, the accumulation of metabolite increased linearly with time, the substrate and metabolites were accurately detected, and the

Table 3. Observed versus predicted PK data (AUC, C_{max} and T_{max}) of tolbutamide in the PBPK model of tolbutamide study.

CL_{int} (μL/min per milligram)	AUC (ng/mL·h)		C_{max} (ng/mL)		T_{max} (h)		References/Comments
	Predicted	Observed[32]	Predicted	Observed[32]	Predicted	Observed[32]	
6.80	453399		34671		3.37		Bio-system for 200 μmol/L
6.00	508844		36079		3.37		Bio-system for 100 μmol/L
4.80	620661	483223	38564	33500	3.87	4.1	Bio-system for 50 μmol/L
5.87	519080		36331		3.62		Bio-system for Mean
2.28	1105571		45984		4.83		[40]
1.87	1256410		47719		5.33		[39]

Table 4. The observed and simulated CL for tolbutamide.

Parameter	Tolbutamide		References/Comments
	Value	Mean	
CL (L/h)	1.05		[33]
	0.79	0.84	[33]
	0.80		[33]
	0.75		[37]
	0.73		[38]
	0.79		[36]
	0.85		[34]
	0.93		[35]
	0.34	0.38	Traditional incubation
	0.42		Traditional incubation
	1.26		Bio-system for 200 μmol/L
	1.11	1.09	Bio-system for 100 μmol/L
	0.89		Bio-system for 50 μmol/L

data could be fitted with a high degree of correlation. Finally, we also sought to further verify whether the system can be applied to and promoted in different species to provide the basis for further optimization and application of the system.

In this Bio-PK metabolic system, HLMs were encapsulated in the FAB hydrogel as a metabolic element. To better monitor the drug concentration in the FAB hydrogels in real-time, microdialysis[43], a semi-invasive sampling technique whose principle is dialysis, was integrated into the Bio-PK system. For analysis of the small sample volumes with low concentrations of analytes frequently present in microdialysis samples[44], LC-MS/MS was used to measure analyte concentrations in the microdialysates[45]. The free drug is more likely to reach the target organ from the vascular compartment and is considered the pharmacologically active portion. Furthermore, plasma and microsomal protein binding plays an important role in pharmacokinetic processes, especially in the distribution and elimination of drug[46]. The main advantage of microdialysis is to exclude proteins from the microdialysate, which stops enzymatic degradation, terminates microsome reactions, and makes sample preparation redundant. Therefore, all microdialysis characteristics are very suitable for use in the Bio-PK metabolic system and will further refine the system.

In fact, the dialysis process is not in complete equilibrium, and the concentration of the drug in the dialysate obtained by microdialysis is different from that in the periprobe fluid. It is mainly the concentration of the drug around the semipermeable membrane of the probe. Thus, the *in vitro* recovery of the probe was used to calibrate the concentration difference. Microdialysis probe recovery was calibrated using the no-net-flux method. No difference in recovery values was observed for the different concentrations of tolbutamide, indicating that the recoveries from the microdialysis probes in the FAB hydrogel are independent of tolbutamide concentration. The recovery of 4-hydroxytolbutamide in the FAB hydrogel was higher than that of tolbutamide, which may be attributed to the metabolism of xenobiotics. This metabolism usually results in compounds that are more polar than the parent drug (reduction in logD), which tends to increase the affinity for drug perfusate[47]. The perfusate of the microdialysis probe is typically aqueous, such as phosphate buffer (PBS), Ringer's solution and anticoagulant citrate dextrose (ACD solution)[48]. Therefore, microdialysis technology is more suitable for the sampling of polar components, and it is difficult to the high hydrophobic and protein binding components[49, 50]. Thus, it is reasonable to expect that 4-hydroxytolbutamide can more easily pass through the membrane compared to tolbutamide.

The average fasted-state hepatic blood flow rate in humans is 90 L/h (1.5 L/min). This increases in the fed state to 120 L/h (2 L/min)[51]. When such a high flow rate is applied to *in vitro* studies, it leads to a higher requirement for the pump; importantly, the FAB hydrogel can be easily destroyed, which may easily lead to the loss of enzyme activity. Thus, a suitable flow rate for the system must be determined. This flow rate should ensure that the FAB hydrogel is not destroyed and that microsomal proteins are firmly locked inside the FAB hydrogel and remain stable for a long period. The release of HLM protein from the FAB hydrogel under different flow rates shows that the HLMs are substantially encapsulated in the FAB hydrogel when the flow rate is less than or equal to 21 mL/min. The formation of metabolites further supports the encapsulation capacity and integrity of the FAB hydrogels. Although the FAB hydrogel remains stable and has good loading capacity at 42 mL/min in the short term, the HLMs will still be washed out over time. Thus, an average flow rate of 10 mL/min was used in the Bio-PK metabolic system to study human metabolism.

Quantitative analysis of the liver metabolism reaction was demonstrated by combining a mathematical modeling approach and experimental analysis. These complex dynamic processes for the Bio-PK metabolic system, including diffusion, microsomal protein binding, transport and reaction and the substrate interchange between the FAB hydrogel and the circular medium, were described. However, traditional *in vitro* systems cannot reproduce this aspect of liver metabolism. A diffusion coefficient, D, is used to calculate the amount of drug in the hydrogel at each time step. Partitioning is assumed to be instantaneous, and the diffusion coefficient can be estimated from the experiment. If diffusion is high, the amount of drug that partitions into the hydrogel will be limited by the blood flow rate (perfusion rate) through the hydrogel. In the dynamic system, tolbutamide quickly diffused in the hydrogel, and its metabolic clearance was far below the blood flow rate. As mentioned above, the flow rate of the Bio-PK metabolic system ensures that the FAB hydrogel is intact and well encapsulated for HLMs; thus, f_m is assumed to be 1 in the model. An average CL_g of 0.0088 mL/min was fitted. The intrinsic clearance was integrated into a PBPK model to predict the PK profile and *in vivo* clearance in humans.

A mini-PBPK distribution model and a first-order absorption model were used for a good description of the tolbutamide PK profile. A distribution of 0.105 L/h was estimated using the

mechanistic tissue composition equation[26] set in Simcyp. This result was in good agreement with the observed results (mean V_{ss}=0.103 L/h)[34-36]. In human beings, tolbutamide is metabolized almost exclusively by CYP2C9 to hydroxytolbutamide. Clearance of tolbutamide from the body is equal to metabolic clearance plus the renal clearance of unchanged drug[32, 35, 52]. Thus, in the PBPK model, elimination was included in the integration of *in vitro* intrinsic clearance and renal clearance. Based on the *in vitro* data and the mechanisms mentioned above, an f_e (fraction of total body clearance via renal excretion) of 0.26% and an f_m (fraction of substrate clearance due to each specific enzyme) of 99.74 were reasonably predicted by the PBPK model, which integrated the average intrinsic clearance from the Bio-PK metabolic system. Studies have shown that the f_m for tolbutamide is 1[53]. The urinary excretion of tolbutamide at different times is also reasonably predicted. The predicted cumulative urinary recovery of tolbutamide was 1.3 μmol between 0 and 6 h (observed 1.5 μmol), 2.4 μmol between 0 and 12 h (observed 2.3 μmol), and 3.3 μmol between 0 and 24 h (observed 3.6 μmol)[54].

The liver plays an important role in the human body and is involved in most of the metabolism and elimination of drugs. On this basis, the system was designed and developed and may be more suitable for a drug whose metabolism mainly occurs in the liver, based on the current research. For drugs metabolized in the intestine or other organs, this system may also achieve simulation and prediction by replacing the corresponding microsomes entrapped in the FAB hydrogels, but this hypothesis still requires further validation. For drugs whose renal elimination and transporters play an important role in disposition or for macromolecular drugs with a limited diffusion rate, the current system may require further optimization and integration of additional mechanisms. In brief, the system tries to better model drug metabolism from the perspective of *in vitro* dynamics. However, the metabolic behavior of the drug is very complicated, and the system must be further refined and optimized, especially for drugs with a complicated metabolic mechanism.

In summary, a dynamic *in vitro* metabolic system based on peristaltic pumps and human liver microsomal encapsulated FAB hydrogel was constructed. The integration and application of microdialysis further improved the system. Compared to the traditional incubation method, more reasonable PK profile and *in vivo* clearance were predicted by the PBPK model integrated with the intrinsic clearance obtained from the Bio-PK system. The successful prediction maximized confidence in the metabolic reaction prediction obtained using the Bio-PK metabolic system, demonstrating the feasibility of applying the dynamic system to screen compounds and to understand the metabolic mechanisms, especially for metabolism involving human study. This novel dynamic system also has the potential to further develop *in vitro* dynamic drug-drug interaction studies. A reliable *in vitro* dynamic DDI model in the human is also highly desired. This research provides a basis and support for later development and research.

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Author contribution

Cai-fu XUE, Wei-min CAI and Bin ZHU designed the research; Cai-fu XUE, Zhe ZHANG and Yan JIN performed the research and analyzed the data; Cai-fu XUE and Zhe ZHANG wrote the paper; Wei-min CAI, Guo MA, Xiao-qiang XIANG and Jun-fen XING reviewed and revised the paper.

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