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Effects of icaritin on cytochrome P450 enzymes in rats

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The purpose of this study was to find out whether icaritin influences the effect on rat cytochrome P450 (CYP) enzymes (CYP1A2, CYP2C9, CYP2E1 and CYP3A4) using cocktail probe drugs *in vivo*. A cocktail solution at a dose of 5 mL/kg, which contained phenacetin (20 mg/kg), tolbutamide (5 mg/kg), chlorzoxazone (20 mg/kg) and midazolam (10 mg/kg), was orally administered to rats treated with multiple doses of icaritin. Blood samples were collected at a series of time-points and the concentrations of probe drugs in plasma were determined by HPLC-MS/MS. The corresponding pharmacokinetic parameters were calculated by the software of DAS 2.0. Treatment with multiple doses of icaritin had inhibitive effects on rat CYP1A2, CYP2C9 and CYP3A4 enzyme activities. However, icaritin has no inductive or inhibitory effect on the activity of CYP2E1. Therefore, caution is needed when icaritin is co-administered with some CYP1A2, CYP2C9 or CYP3A4 substrates, which may result in treatment failure and herb-drug interactions.

1. Introduction

"*Herba Epimedii Sagittatum* (common name in Chinese Yin Yang Huo)", the dried aerial parts of genus *Epimedium* species (family Berberidaceae), are used as a tonic, aphrodisiac and anti-rheumatic medicine in China, and are commonly used for the treatment of cardiovascular diseases and osteoporosis, and in improving sexual and neurological functions (Sze et al. 2010). Icaritin ($C_{21}H_{20}O_6$, molecular weight: 368 g/moL) is recognized as the major active monomer refined from the Chinese herb *Herba Epimedii Sagittatum* (Chen et al. 2008; Shen et al. 2007; Wu et al. 2003). As a highly interesting natural flavonoid compound for drug development, icaritin has been shown to possess many pharmacological and biological activities, including inhibition of human endometrial cancer cells (Tong et al. 2011), breast cancer cells (Guo et al. 2011), human prostate carcinoma PC-3 cells (Huang et al. 2007b), and chronic myeloid leukemia (Zhu et al. 2011). In addition, it could suppress the osteoclasts differentiation (Huang et al. 2007a) and prevent steroid-associated osteonecrosis (Zhang et al. 2009), promote cardiac differentiation of mouse embryonic stem (ES) cells *via* reactive oxygen species (ROS) signaling (Wo et al. 2008), and stimulate neuronal differentiation (Wang et al. 2009). However, the effect of icaritin on the cytochrome P450 (CYP) remains to be elucidated.

The CYP system involved in drug metabolism in humans are predominantly expressed in the liver and intestine. They are membrane proteins bound to the endoplasmic reticulum. The CYPs represent a superfamily of Phase I heme-thiolate oxidative enzymes. It is believed that more than 10 CYPs in the human liver are involved in drug metabolism. Among the various CYP isozymes, CYP1A2, CYP2C9, CYP2E1, and CYP3A4 are considered to be most important (Zhou et al. 2009). Each enzyme can metabolize a vast array of xenobiotics.

CYPs are a superfamily of mixed function oxidases that are responsible for the metabolism of many drugs and represent the major site for drug-drug and herb-drug interactions. Inhibition of CYPs can lead to clinically relevant increases in the exposure of the affected drug and thus to increased toxicity (Izzo and Ernst 2009; Pelkonen et al. 2008). Further, some of the CYP isozymes are also subject to induction by xenobiotics *via* activation of nuclear receptors, with decreased exposure of the affected compound leading to therapeutic failure or toxicological implications due to higher levels of a toxic metabolite (Izzo and Ernst 2009; Park et al. 1996; Pelkonen et al. 2008). Moreover, many carcinogens are metabolized by CYPs to either biological inactive metabolites or to chemically reactive electrophilic metabolites that covalently bind to DNA resulting in carcinogenicity (Conney 2003). Since many chemical carcinogens are metabolized by CYPs to both inactive, as well as to carcinogenic metabolites, the effects of inducers of these enzymes on the carcinogenicity of a chemical will depend on the inducer's effects on the different metabolic pathways.

A number of natural products have been demonstrated to modulate CYPs, including the induction of specific CYP isoforms, and the activation or inhibition of these enzymes (Izzo and Ernst 2009; Johnson 2008; Saxena et al. 2008). For a new molecular entity, it is important to assess its possible inhibitory or inductive effects on CYP enzymes.

Evaluation of the effect of herbs or new drugs on CYP enzyme activities is essential in clinical development as it may explain inter-subject variability, investigate potentially toxic effects and predict drug-drug interactions. The CYP specific probe drugs can be used to determine the real time activities of important drug-metabolizing enzymes. Compared to the administration of a single specific probe in multiple studies, the "cocktail" approach can give information on several CYP activities of several pathways in a single experiment (Breimer 1983). Several different cocktails of markers have been used and many cocktail methods have been developed and evaluated. However, the disadvantages of this cocktail approach are also well defined: the frequent occurrence of probe drug

side-effects (*in vivo* use), more sample consumption, more time consumption and complicated analytical methods (Tanaka et al. 2003). Nevertheless, the cocktail approach is widely used to assess the activities of CYP isoforms and is now one of the basic analytical tools in initial drug evaluation after developing precise analytical methods (Zlokarnik et al. 2005).

Interaction of herbal products with drugs represents a major safety concern for drugs with narrow therapeutic indices or in patients receiving drug treatment for chronic illness/disease (Hu et al. 2005; Izzo et al. 2005). Induction or inhibition of hepatic and intestinal drug metabolizing enzymes represent the major mechanism for the enhanced or reduced bioavailability of drugs when herbal/botanical products are co-administered (Zhou et al. 2003). For patients receiving multiple drug regimens for treatment of diseases, potential herb-drug interaction may be important. Despite the popular use of icaritin, often as one of the components of multiple herb formulas, the potential of these compounds in affecting hepatic drug metabolism have not been investigated. In this study, the effects of icaritin on the metabolism of probe substrates of CYP isoforms including CYP1A2, CYP2C9, CYP2E1 and CYP3A4, which are important in the metabolism of a variety of xenobiotics were investigated by using a cocktail method in rats.

2. Investigations and results

A validated HPLC-MS/MS method was used to determine the levels of the four probe drugs (phenacetin for CYP1A2, tolbutamide for CYP2C9, chlorzoxazone for CYP2E1 and midazolam for CYP3A4) in rat plasma after multiple doses of icaritin.

2.1. Effect of icaritin on rat activity of CYP1A2

Pharmacokinetic profiles of phenacetin after icaritin treatment were used to describe the activity of CYP1A2. The effects of different dose treatment groups of icaritin on pharmacokinetic parameters of phenacetin in rats are presented in Table 1. Mean plasma concentration-time curves of phenacetin in different groups are presented in Fig. 1. After pretreatment with multiple doses of icaritin, the $t_{1/2}$, T_{max}, C_{max}, $AUC_{(0-\infty)}$ and $MRT_{(0-\infty)}$ of phenacetin were increased significantly compared to those of the corresponding control group, CL of phenacetin was decreased significantly. The results indicate that metabolism of phenacetin in these treatment groups was evidently slowed down, and icaritin had the potential to inhibit rat hepatic CYP1A2 activity *in vivo*.

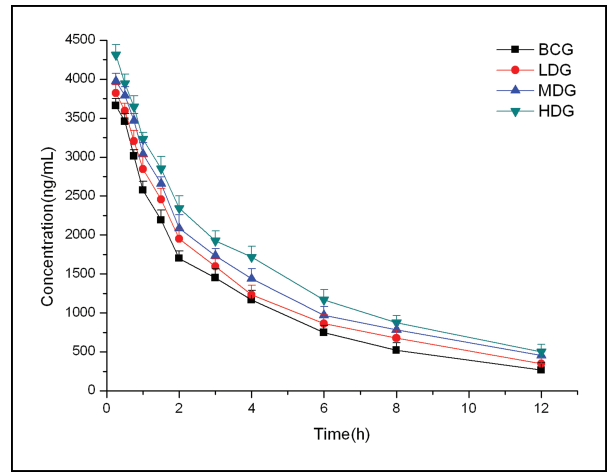

Fig. 1: Mean plasma concentration-time curves of phenacetin in rats.

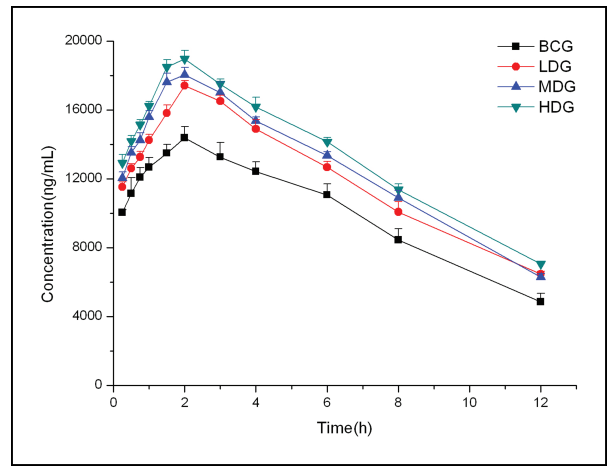

Fig. 2: Mean plasma concentration-time curves of tolbutamide in rats.

2.2. Effect of icaritin on rat activity of CYP2C9

CYP2C9 activity was evaluated by comparing pharmacokinetic behaviors of tolbutamide between the control group and different icaritin treatment groups. The main pharmacokinetic parameters of tolbutamide are listed in Table 2. Mean plasma concentration-time curves of tolbutamide in different groups are presented in Fig. 2. The results show that after pretreatment with icaritin (especially high doses), the $t_{1/2}$, T_{max}, C_{max}, $AUC_{(0-\infty)}$ and $MRT_{(0-\infty)}$ of tolbutamide were increased significantly compared to those of the corresponding control group, CL of tolbutamide was decreased significantly. These results demonstrated that CYP2C9 activity was significantly inhibited by icaritin in rats.

2.3. Effect of icaritin on rat activity of CYP2E1

The effects of different treatment groups of icaritin on pharmacokinetic parameters of chlorzoxazone in rats are presented in Table 3. Mean plasma concentration-time curves of chlorzoxazone in different groups are presented in Fig. 3. The pharmacokinetic parameters ($t_{1/2}$, T_{max}, C_{max}, $AUC_{(0-\infty)}$, $MRT_{(0-\infty)}$ and CL) of chlorzoxazone in rats showed no

Table 1: Main pharmacokinetic parameters of phenacetin after multiple doses of icaritin in rat plasma (n = 6, Mean ± SD)

Parameter	BCG	LDG	MDG	HDG
$t_{1/2}$ (h)	3.794 ± 0.457	4.347 ± 0.137	5.096 ± 0.404	5.644 ± 0.656
T_{max}(h)	0.234 ± 0.003	0.253 ± 0.003	0.254 ± 0.004	0.263 ± 0.004
C_{max} (ng/mL)	3661.549 ± 216.049	3821.978 ± 231.772	3971.835 ± 327.723	4312.217 ± 357.257
$AUC_{(0-\infty)}$(μg·h/L)	14057.467 ± 328.97	16510.018 ± 280.29	19274.111 ± 298.192	21057.372 ± 561.233
$MRT_{(0-\infty)}$(h)	4.982 ± 0.347	5.719 ± 0.360	6.621 ± 0.279	6.778 ± 0.605
CL/F (L/h/kg)	1.423 ± 0.033	1.211 ± 0.026	1.038 ± 0.016	0.950 ± 0.025

* Significantly different from control, $P < 0.05$; ** Significantly different from control, $P < 0.01$.

Table 2: Main pharmacokinetic parameters of tolbutamide after multiple doses of icaritin in rat plasma (n = 6, Mean ± SD)

Parameter	BCG	LDG	MDG	HDG
$t_{1/2}$ (h)	5.989 ± 1.763	6.935 ± 1.142	7.269 ± 1.466	7.427 ± 1.271
T_{max}(h)	2.286 ± 0.756	2.543 ± 0.869	2.929 ± 0.689	3.230 ± 0.893
C_{max} (ng/mL)	14672.125 ± 415.031	17413.924 ± 288.707	18093.430 ± 448.996	18957.924 ± 592.412
$AUC_{(0-\infty)}$(μg·h/L)	162835.087 ± 13429.52	212237.514 ± 19328.530	228526.348 ± 23931.761	229472.811 ± 23822.740
$MRT_{(0-\infty)}$(h)	9.305 ± 1.986	10.651 ± 1.528	11.089 ± 1.927	11.350 ± 1.677
CL/F (L/h/kg)	0.031 ± 0.003	0.024 ± 0.002	0.022 ± 0.002	0.021 ± 0.001

* Significantly different from control, $P < 0.05$; ** Significantly different from control, $P < 0.01$.

Table 3: Main pharmacokinetic parameters of chlorzoxazone after multiple doses of icaritin in rat plasma (n = 6, Mean ± SD)

Parameter	BCG	LDG	MDG	HDG
$t_{1/2}$ (h)	4.999 ± 0.287	5.245 ± 0.216	5.323 ± 0.131	5.004 ± 0.163
T_{max}(h)	3.124 ± 0.121	2.983 ± 0.241	3.091 ± 0.212	3.231 ± 0.330
C_{max} (ng/mL)	10034.429 ± 128.965	9748.714 ± 110.845	10388.714 ± 169.152	10190.143 ± 155.694
$AUC_{(0-\infty)}$(μg·h/L)	101637.285 ± 1669.296	102488.977 ± 2040.828	99654.440 ± 1264.273	98147.844 ± 1528.863
$MRT_{(0-\infty)}$(h)	7.930 ± 0.407	8.364 ± 0.293	7.570 ± 0.254	7.913 ± 0.218
CL/F (L/h/kg)	0.199 ± 0.002	0.195 ± 0.004	0.213 ± 0.001	0.204 ± 0.003

*Significantly different from control, $P < 0.05$; ** Significantly different from control, $P < 0.01$.

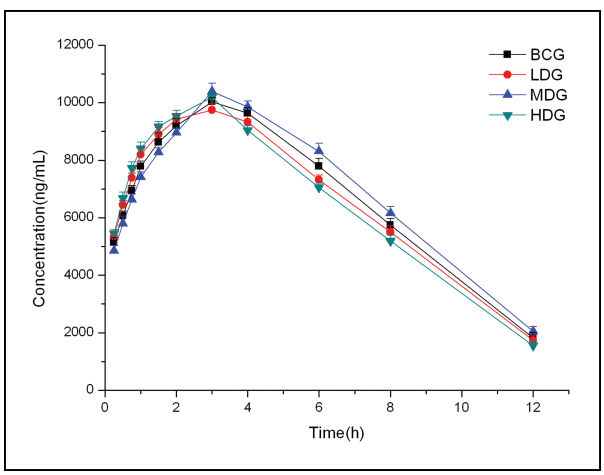

Fig. 3: Mean plasma concentration-time curves of chlorzoxazone in rats.

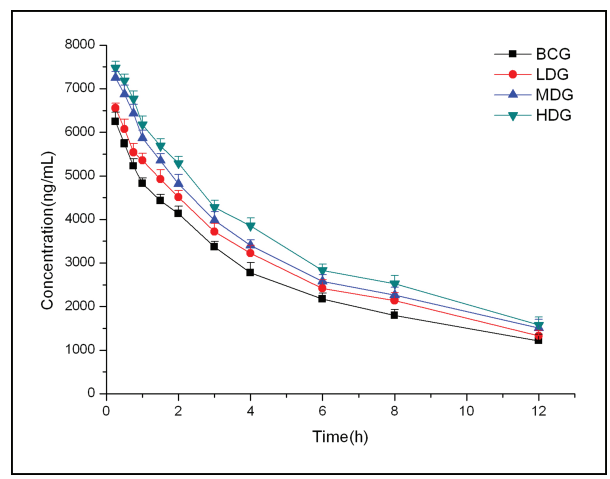

Fig. 4: Mean plasma concentration-time curves of midazolam in rats.

significant difference between different treatment groups and the control group after pretreatment with multiple doses of icaritin. Therefore, the pharmacokinetic behaviors of chlorzoxazone indicated that icaritin did not affect rat hepatic CYP2E1 activity *in vivo*.

2.4. Effect of icaritin on rat activity of CYP3A4

CYP3A4 activity was evaluated by comparing the pharmacokinetic behavior of midazolam between the control group and different icaritin treatment groups. The effects of icaritin on pharmacokinetic parameters of midazolam in rats are presented in Table 4. Mean plasma concentration-time curves of midazolam in different groups are presented in Fig. 4. After pretreatment with multiple doses of icaritin, the pharmacokinetic parameters ($t_{1/2}$, T_{max}, C_{max}, $AUC_{(0-\infty)}$ and $MRT_{(0-\infty)}$) of midazolam in rats were increased significantly compared to those of the corresponding control group, CL of midazolam was decreased significantly. The results indicated that icaritin had the potential to inhibit rat hepatic CYP3A4 activity *in vivo*.

3. Discussion

Multiple drug therapy is common therapeutic practice, particularly in patients with several diseases or in complex conditions. Herbal medicines have received increasing interest as an alternative to the present therapeutic practice, and the demand for concomitant use of herbal medicines and drugs has currently increased De Smet 2002, which emphasizes the importance and urgency of examining the drug interactions between natural products and chemical drugs. In addition, many preparations of herbal medicines are available over-the-counter, and these preparations can be self-prescribed without recommendation or advice provided by a physician. In fact, many herbal medicines such as icaritin are regarded to show neither side-effects nos drug-drug interactions. In addition, herbal medicines are not required to undergo careful and scientifically rigorous examinations of safety that are required for conventional pharmaceutical products. Due to these reasons, more and more physicians and pharmacists are concerned about herb-drug interactions. Modulation of drug-metabolizing enzymes is one of the main causes of herb-drug interactions (Tanaka 1998).

Table 4: Main pharmacokinetic parameters of midazolam after multiple doses of icaritin in rat plasma (n = 6, Mean ± SD)

Parameter	BCG	LDG	MDG	HDG
$t_{1/2}$ (h)	6.726 ± 0.876	7.389 ± 0.753	8.069 ± 1.074	8.222 ± 0.940
T_{max}(h)	0.212 ± 0.004	0.246 ± 0.003	0.251 ± 0.003	0.262 ± 0.004
C_{max} (ng/mL)	6244.784 ± 111.747	6556.714 ± 117.088	7249.571 ± 146.065	7479.571 ± 148.010
$AUC_{(0-\infty)}$(μg·h/L)	42752.797 ± 2204.681	47234.045 ± 1549.308	53192.901 ± 1423.839	55191.705 ± 1579.586
$MRT_{(0-\infty)}$(h)	9.152 ± 1.003	9.687 ± 0.874	9.876 ± 1.291	10.780 ± 0.964
CL/F (L/h/kg)	0.234 ± 0.002	0.212 ± 0.003	0.189 ± 0.001	0.181 ± 0.005

*Significantly different from control, $P < 0.05$; ** Significantly different from control, $P < 0.01$.

Recently, we developed a novel four probe-drug cocktail, consisting of phenacetin, tolbutamide, chlorzoxazone and midazolam, in order to establish and optimize a simplified, rapid, selective HPLC-MS/MS method to determine four probe drugs in a single run for the evaluation of CYP1A2, CYP2C9, CYP2E1 and CYP3A4 activities. The determination of the novel "cocktail" was validated in terms of recovery, linearity, precision and accuracy and applied to the analysis of plasma samples in rats. Therefore, in the present study, we investigated the effect of icaritin on the activities of four major CYP isozymes (CYP1A2, CYP2C9, CYP2E1 and CYP3A4) in rats.

According to our results, CYP1A2 activity was significantly inhibited by icaritin after multiple administrations in rats. The differences among the three doses of icaritin suggest that the subtypes of enzymes affected did correlate with the dose of administration. As we know, CYP1A2 accounts for about 13% of the total CYP content in human liver (Shimada et al. 1994) and is involved in the metabolism of several endogenous compounds and some widely used drugs, also it could activate procarcinogens such as aflatoxin B1, a commonly recognized hepatocarcinogen (Mustajoki et al. 1994). The above results show that when icaritin is used in combination with other drugs which are metabolized by CYP1A2, potential herb-drug interactions should be paid more attention to reduce adverse reactions.

CYP2C9 is one of the most abundant CYP enzymes in the human liver (∼20% of hepatic total CYP content), where it metabolizes approximately 15% of clinically used drugs (>100 drugs), including a number of drugs with narrow therapeutic ranges (Miners and Birkett 1998). In addition, CYP3A4 was known as the rate-limiting step in the metabolism and clearance of a large variety of clinical medications, including many pediatric drugs (Lu et al. 2003). Therefore, induction or inhibition of CYP2C9 and CYP3A4 may lead to undesirable effects. According to our results, both CYP2C9 and CYP3A4 activity could be significantly inhibited by icaritin after multiple administrations in rats. Thus, under co-administration with icaritin, drugs metabolized by human CYP2C9 and CYP3A4 may need dose adjustment to avoid an undesirable herb-drug interaction.

CYP2E1 has a unique capacity to activate many xenobiotics to hepatotoxic or carcinogenic products. CYP2E1 is responsible for the metabolism of a large number of low-molecular-weight chemicals, such as aliphatic, aromatic, and halogenated hydrocarbons (Yao et al. 2011). Due to its ability to metabolize the compounds, CYP2E1 may be an important determinant factor of humans' susceptibility to toxicity and carcinogenicity of industrial and environmental chemicals. In our study, we found that there was no significant difference of the pharmacokinetic parameters of chlorzoxazone before and after administration of icaritin. This suggests that icaritin has no inductive or inhibitory effect on the activity of CYP2E1 after a long period of administrations in rats.

In conclusion, the inconspicuous effect of icaritin on CYP2E1 suggests that there are no clinically relevant herb-drug interactions between the drugs metabolized by this enzyme and icaritin when they are used concomitantly. In addition, from our present results, we cannot exclude that comedication of icaritin with drugs metabolized by CYP1A2, CYP2C9 and CYP3A4 may inhibit metabolism and increase plasma concentrations of these drugs, which will result in herb-drug interactions. Further clinical studies are required to fully assess the safety of icaritin in terms of CYP.

4. Experimental

4.1. Chemicals and reagents

Icaritin (98.0% purity) was purchased from Shanghai Ronghe Pharmaceutical Science Co Ltd. (Shanghai, China). Phenacetin (purity > 98.0%), tolbutamide (purity > 98.0%), chlorzoxazone (purity > 98.0%), midazolam (purity > 98.0%) and the internal standard carbamazepine (IS, purity > 98.0%) were also purchased from Sigma-Aldrich Company (St. Louis, USA). HPLC grade acetonitrile and methanol were from Merck Company (Darmstadt, Germany). All other chemicals were of analytical grade and used without further purification. Ultra-pure water (resistance > 18.2 mΩ) prepared by a Millipore Milli-Q purification system (Bedford, USA).

4.2. Apparatus

All analyses were performed with a 1200 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler, a thermostatted column compartment, and a Bruker Esquire HCT mass spectrometer (Bruker Technologies, Bremen, Germany) equipped with an electrospray ion source and controlled by ChemStation software.

4.3. Animals

Male Sprague-Dawley rats with body weights of 220 ± 30 g were provided by the Animal Care and Use Committee of Wenzhou Medical College. They were housed in cages at 23–25 °C and had free access to regular rodent diet and water. After an 1-week acclimatization period, the rats were used for experiments and all efforts were made to minimize any animal suffering. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Wenzhou Medical College and were in accordance with the Guide for the Care and Use of Laboratory Animals.

4.4. Drug administration and sampling

Twenty four male Sprague-Dawley rats were randomly divided into 4 groups (total 24 rats, n = 6): blank control group (BCG), low dosage group (LDG, 1.0 mg/kg), medium dosage group (MDG, 4.0 mg/kg) and high dosage group (HDG, 16.0 mg/kg), which were given vehicle or icaritin (dissolved in 0.5% CMC-Na solution) once daily. After oral administration for consective 14 days, a cocktail solution at a dose of 5 mL/kg, which contained phenacetin (20 mg/kg), tolbutamide (5 mg/kg), chlorzoxazone (20 mg/kg) and midazolam (10 mg/kg) in CMC-Na solution, was administered orally to all rats in each group. Blood samples of each rat were collected pre-dose (0 h) and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12 h after probe drugs administration and immediately separated by centrifugation at 13,000 rpm for 10 min to obtain plasma. From the 7th blood collection, the rats were treated by oral administration of normal saline of the same blood collection volume in order to restore blood capacity quickly. 100 μL plasma samples were transferred to another tube and stored frozen at –80 °C until analyzed.

4.5. Sample preparation

In a 1.5 mL centrifuge tube, aliquot of 0.2 mL acetonitrile with carbamazepine (500 ng/mL) as the internal standard was added to 0.1 mL of

collected plasma sample. After the tube was vortex-mixed for 1.0 min, the sample was centrifuged at 13,000 rmp for 10 min. Next, the supernatant (10 μL) was injected into the HPLC-MS/MS system for analysis.

4.6. Chromatographic conditions

Chromatographic separation was achieved on an Agilent Zorbax SB-C18 column (150 mm × 2.1 mm, 3.5 μm) with the column temperature set at 30 °C. The mobile phase consisted of (A) acetonitrile and (B) 0.1% formic acid in water, and a gradient elution of 10–85% A at 0–1.5 min, 85–85% A at 1.5–6.0 min, 85–10% A at 6.0–7.0 min and 10–10% A at 7.0–10.0 min was employed. The flow rate was 0.4 mL/min. The injection volume was 10 μL.

The quantification was performed by the peak-area method. The determination of target ions were performed in SIM mode (m/z 180 for phenacetin, m/z 271 for tolbutamide, m/z 168 for chlorzoxazone, m/z 326 for midazolam and m/z 237 for IS) and positive ion electrospray ionization interface. Drying gas flow was set to 6 L/min and temperature to 350 °C. Nebuliser pressure and capillary voltage of the system were adjusted to 20 psi and 3,500 V, respectively.

4.7. Statistical analysis

The concentration-time profile of each probe drug was analyzed by DAS software (Version 3.0, Wenzhou Medical College, China) and statistic analyses were tested by t-test using SPSS (Version 13.0, Wenzhou Medical College, China). A value of $P < 0.05$ was considered to be statistically significant.

Conflict of interest: The authors have declared that there is no conflict of interest.

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