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Short communication

Determination of propofol UDP-glucuronosyltransferase (UGT) activities in hepatic microsomes from different species by UFLC–ESI-MS

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ABSTRACT

Propofol *O*-glucuronidation has been used as probe reaction to phenotype UGT1A9 activity in human liver, thus a sensitive and specific method for determination of propofol *O*-glucuronide (PG) is urgently desirable. In the current study, a new LC–ESI-MS method for determination of PG in hepatic microsomes from human (HLM), monkey (CyLM), dog (DLM), minipig (PLM), rat (RLM) and mouse (MLM) was developed and validated using 4-methylumbelliferyl-β-D-glucuronide as an internal standard (IS). PG and IS was separated by a Shim-pack XR-ODS column (100 mm × 2.0 mm, 2.2 μm, Shimadzu) under gradient conditions with the mobile phase of acetonitrile and water containing 0.2% acetic acid (v/v). The mass spectrometric detection was performed under selected ion monitoring (SIM) for PG at *m*/*z* 353 and IS at *m*/*z* 351. The assay exhibited linearity over the range 0.05–30 μM for PG with the correlation coefficient of 0.9995. The intra- and inter-day precision was less than 7.2%, with accuracy in the range 93.8–107.5%. The developed method was successfully used for characterizing interspecies and human individual differences in the *O*-glucuronidation activity towards propofol, as well as investigating inhibitory effects of androsterone and phenylbutazone on propofol *O*-glucuronidation in HLM.

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1. Introduction

Metabolic information, such as metabolic pathway, metabolizing enzymes kinetics, and metabolic interactions is very helpful to elucidate the mechanism of action-process for a given drug [1]. Due to the ethical constraints, animals often replace human in drug metabolism and toxicity studies. However, species-dependent variations in drug metabolism and clearance always produce species-specific effect and toxicity. In order to obtain reliable extrapolation between animal model(s) and human, it is proposed that those animals displaying similar metabolic patterns with human are preferred [2].

UDP-glucuronosyltransferases (UGTs, EC 2.4.1.17), a superfamily of membrane-bound enzymes, play an important role in metabolic elimination of enobiotics, and xenobiotics *via* glucuronidation. About 45 UGT isozymes are known in mammals, and these UGTs can be classified into two families (UGT1 and UGT2) based on their sequence identities [3]. Until now, the information relevant to the activity of UGT isoenzyme(s) among different species is insufficient, though it is very critical to select suitable animal model(s) for pharmacokinetic, toxicological and pharmacological studies. Furthermore, it has been reported that human UGT enzymes can be greatly affected by the environmental, genetic and other factors, therefore characterizing the magnitude of interindividual differences is important to risk prediction and dose regulation in drug discovery and clinical applications [4].

A common strategy for investigation of activity of a given UGT enzyme is to monitor the metabolite(s) formation of specific probe substrate(s). However, most UGTs have broad and overlapping substrate specificities, while high selective substrates of UGT isoform(s) have been rarely reported. Propofol, a short-acting intravenous anesthetic, is predominantly metabolized by human UGT1A9 (resulting in PG) and can serve as a substrate probe to phenotype the activities of this enzyme [5]. As one of the most important UGT isoforms in human, UGT1A9 is involved in the glucuronidation of many drugs including bulky phenols, flavonoids and anthraxquinones [3]. Although several analytical methods using high performance liquid chromatography (HPLC) with diode-array detector and scintillation detector [6,7], or using the radioactive [^{14}C] propofol [8] to monitor the formation of PG in human tissues have been reported, these methods suffered from low sensitivity, time-consuming pretreatment and high cost.

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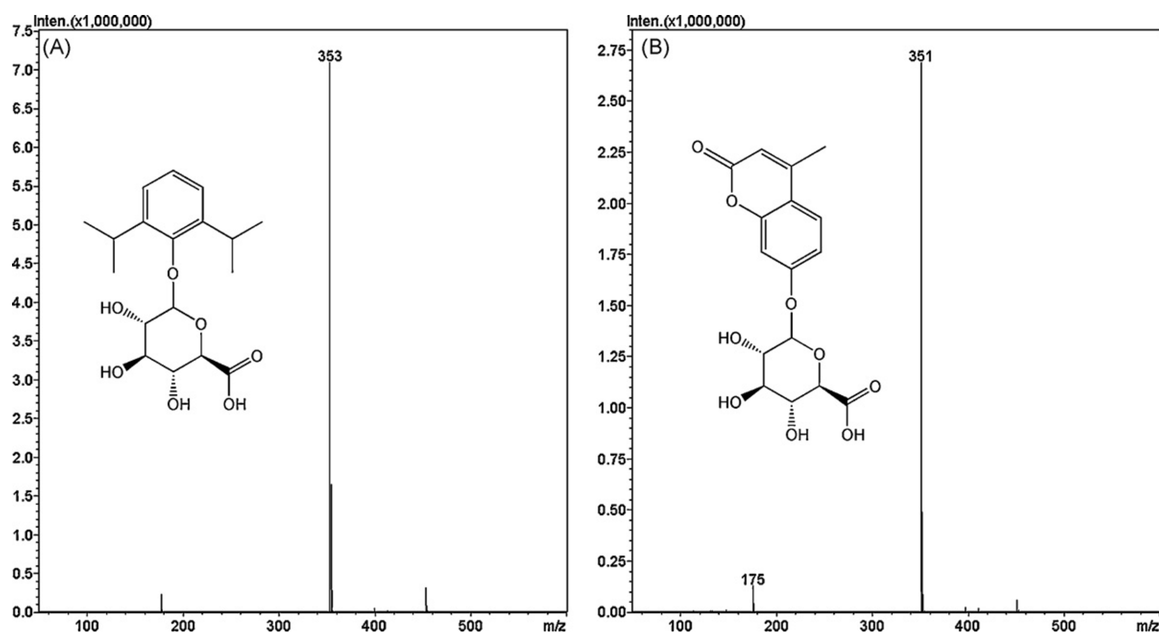

Fig. 1. Chemical structures and MS spectra in negative-ion mode of PG (A) and IS (B).

In this study, a sensitive and specific method incorporating of ultra-fast liquid chromatography (UFLC) with MS detection was developed for screening the propofol *O*-glucuronidation activities in liver-derived preparations from human and animals. The pooled hepatic microsomes from human, monkey, dog, minipig, rat and mouse were used to evaluate the interspecies differences using this important UGT probe reaction. To evaluate human interindividual differences, the propofol *O*-glucuronidation activities in a human liver bank containing 17 individuals were also determined using this method. Furthermore, the method was also applied in chemical inhibition assay for propofol *O*-glucuronidation using HLM as enzyme source.

2. Experimental

2.1. Reagent and chemicals

Propofol, alamethicin, uridine-5′-diphosphoglucuronic acid (UDPGA), magnesium chloride, β-glucuronidase (EC 3.2.1.31), and D-saccharic acid 1,4-lactone were purchased from Sigma (St. Louis, MO, USA). The internal standard (IS) 4-methylumbelliferyl-β-D-glucuronide (≥98%) was purchased from Alfa Aesar (Beijing, China). PG was obtained from Toronto Research Chemicals (North York, Ontario, Canada). All other reagents and solvents were of analytical reagent grade.

2.2. Preparation of hepatic microsomes

Human liver samples (n = 17, male Chinese, ages from 27 to 48), mouse livers from 10 ICR mice (male, weight 21–34 g, 7-week-old) and rat livers from 10 Sprague-Dawley rats (male, weight 180–220 g, 6-week-old) were provided by Dalian Medical University, China. Minipig liver from three Colony-bred Chinese Bama minipigs (male, weight 10–12 kg, 6-month-old) and dog livers from three beagle dogs (male, weight 10–12 kg, 12-month-old) were provided by Third Military Medical University, China. Monkey livers from three Cynomolgus Monkeys (male, weight 2.7–2.9 kg, 4-year-old) were provided by animal center of Chinese Academy of Military Medical Sciences, China. Liver specimens were stored in liquid nitrogen until preparation of microsomes. Microsomes of human and experimental animals were prepared from liver tissue by differential ultracentrifugation according to the methods described by Guengerich [9]. The microsomal protein content was determined according to the Lowry method [10], using bovine serum albumin as standard.

2.3. Propofol glucuronidation

The incubation mixture (200 μl) contained hepatic microsomes (0.5 mg/ml), 5 mM UDPGA, 5 mM $MgCl_2$, 25 μg/ml alamethicin, 10 mM D-saccharic acid 1,4-lactone, propofol (2–1500 μM) and Tris–HCl buffer (pH 7.4). After 20 min of incubation at 37 °C, the reaction was terminated by the addition of 100 μl methanol containing IS, 4-methylumbelliferyl-β-D-glucuronide (final concentration, 40 μM). Deproteined samples were centrifuged at 20,000 × *g* for 10 min at 4 °C to obtain the supernatant for UFLC analysis. Control incubations without UDPGA or without substrate or without microsomes were performed to ensure that the metabolites produced were microsome- and UDPGA-dependent. In order to confirm that the metabolite was glucuronide compound, enzymatic hydrolysis was performed as described previously [11].

Enzyme-inhibition studies were performed using various concentrations (0, 10, 20, 40, 80, 100, 200, 300 μM) of androsterone or phenylbutazone at the propofol concentration of 75 μM in HLM. Inhibition kinetic studies were performed in a range of concentrations of propofol (25, 50, 75 and 150 μM) and different concentrations (0, 40, 80, 160 and 320 μM) of androsterone or phenylbutazone in HLM.

2.4. Instrumentation and analytical conditions

The ultra-fast liquid chromatography (UFLC) spectrometry system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD pumps, a DGU-20A3 vacuum degasser, an SIL-20ACHT autosampler, a CTO-20AC column oven, an SPD-M 20A diode-array detector, a CBM-20A communications bus module, a mass detector (2010EV) with an ESI interface, and a computer equipped with UFLC-MS Solution version 3.41 software.

Table 1
Precision and accuracy of propofol *O*-glucuronide quality control samples ($n = 6$).

Intra-day				Inter-day			
Concentration known (μM)	Concentration found (μM)	Accuracy (%)	Precision CV (%)	Concentration known (μM)	Concentration found (μM)	Accuracy (%)	Precision CV (%)
0.0500	0.0469	93.8	6.7	0.0500	0.0491	98.2	7.2
0.150	0.161	107.3	5.6	0.150	0.158	105.3	2.6
2.00	1.92	96.2	3.3	2.00	2.15	107.5	2.9
20.0	19.7	98.4	5.1	20.0	19.7	98.7	5.2
30.0	30.4	101.2	4.8	30.0	29.8	99.4	4.2

The chromatographic separation was achieved using a Shim-pack XR-ODS column (100 mm × 2.0 mm, 2.2 μm, Shimadzu). The mobile phase consisted of CH_3CN (A) and 0.2% acetic acid (B) with the following gradient program: 0–2 min, 95–83% B; 2–7 min, 83–20% B; 7–9.5 min, 10% B; 9.5–12.5 min, 95% B. Column temperature was kept at 40 °C. The flow rate was set at 0.3 ml/min and the injection volume was 10 μl. The DAD detection was achieved in the range of 190–370 nm, and the maximal absorption wavelengths of PG and IS were 260 and 320 nm, respectively. Mass detection was performed in both positive and negative-ion mode from m/z 100 to 1000. The detector voltage was set at +1.50 kV, and −1.55 kV for positive and negative-ion detection, respectively. For quantification of PG and IS, the SIM mode with negative ESI was chosen because of the specific formation of molecular ion $[M-H]^-$ (Fig. 1). After optimization, the MS detection conditions were set as follows: voltage = 4 kV, interface voltage = 40 V, nebulizing gas (N_2) flow = 1.5 l/min, and the drying gas (N_2) pressure = 0.06 MPa.

2.5. Calibration standards, quality control samples and method validation

PG was dissolved in 50 mM Tris–HCl buffer (pH 7.4) to prepare stock solutions (3 mM). Using these stock solutions, calibration standards were prepared in drug-free and heat-inactivated human hepatic microsomes at nine concentration points (0.05, 0.1, 0.5, 1, 2, 5, 8, 12.5 and 30 μM of PG). Five quality control (QC) samples were prepared in the similar way for method validation. These QC samples were prepared to contain 0.05 μM (QC1), 0.15 μM (QC2), 2 μM (QC3), 20 μM (QC4) and 30 μM (QC5) of PG. A working IS solution was prepared by diluting 4-methylumbelliferyl-β-D-glucuronide stock solution (12 mM) in methanol to obtain 120 μM. All the solutions and QC samples were stored at −20 °C until analysis. The resulting peak area ratios (the analyte/IS) were plotted against the concentrations.

Method validation with respect to specificity, matrix effect, linearity, quantification limit, precision and accuracy, recovery, dilution integrity and stability was performed according to the guideline suggested by U.S. Food and Drug Administration (www.fda.gov/cvm).

2.6. Data analysis

Apparent Michaelis constant (K_m) and maximum velocity (V_{max}) values were estimated by analyzing Eadie–Hofstee plots. The half inhibition concentration (IC_{50}) values were determined graphically. The apparent inhibition constants (K_i) were calculated with nonlinear regression according to the equation for competitive inhibition using Origin (Orgin Lab Corporation, Northampton, MA).

3. Results and discussion

3.1. Confirmation of PG formation

A new peak was detected when propofol (100 μM) was incubated with HLM, CyLM, PLM, RLM, or MLM in the presence of UDPGA, while the peak was absent in the negative controls without UDPGA/substrate/microsomes. No product peak was observed in the incubated samples with DLM, as described by a previous report [12]. The negative ESI mass spectra of this peak showed a deprotonated molecule at m/z 353 ($[M-H]^-$), corresponding to the glucuronide metabolite of propofol (Fig. 1). The LC retention time, UV and MS spectrum of this metabolite agreed well with those of authentic standard. In addition, the metabolite can be hydrolyzed by β-glucuronidase to the parent (data not shown). All of these evidence suggested that the metabolite formed in different hepatic microsomes incubation systems was PG.

3.2. Method development

Highly sensitive and specific analytical methods are preferred in drug metabolism for both qualitative and quantitative analysis [13]. It has been reported that UV detector was more sensitive than fluorescence and electrochemical detector for detecting PG in human plasma and urine samples [6,7]. In the current study, MS-based method was used to quantify the formation of PG, due to its high sensitivity (LLOQ = 0.05 μM) compared with UV-based method (LLOQ = 1 μM). 4-Methylumbelliferyl-β-D-glucuronide was employed as IS for consideration of its similarity ionization behavior (facilitate in the negative-ion) and structure properties (contain the glucuronide) compared with PG. Acetonitrile/acetic acid were finally selected as mobile phase, due to the specific and reproducible formation of the molecular ion $[M-H]^-$ for both PG and IS, by comparison of other mobile phase, such as acetonitrile/water and acetonitrile/formic acid.

3.3. Method validation

PG and IS were well separated under the current chromatographic conditions. No significant interferences from endogenous compounds were observed in HLM (Fig. 2), CyLM, DLM, PLM, RLM or MLM samples. The method was fairly sensitive with a LLOQ of 0.05 μM for PG. Linearity of calibration range (0.05–30 μM) was assessed by weighted ($1/x$) least squares linear regression of prepared and assayed in sextuplicate. The standard curve for PG in HLM was linear over the concentration range of 0.05–30 μM, with coefficient of 0.9995. The precision and accuracy of the method were determined with QC samples on the same day or on six different days. Intra- and inter-assay precision (expressed as %CV) were less than 7.2%, with accuracy in the range 93.8–107.5% (Table 1).

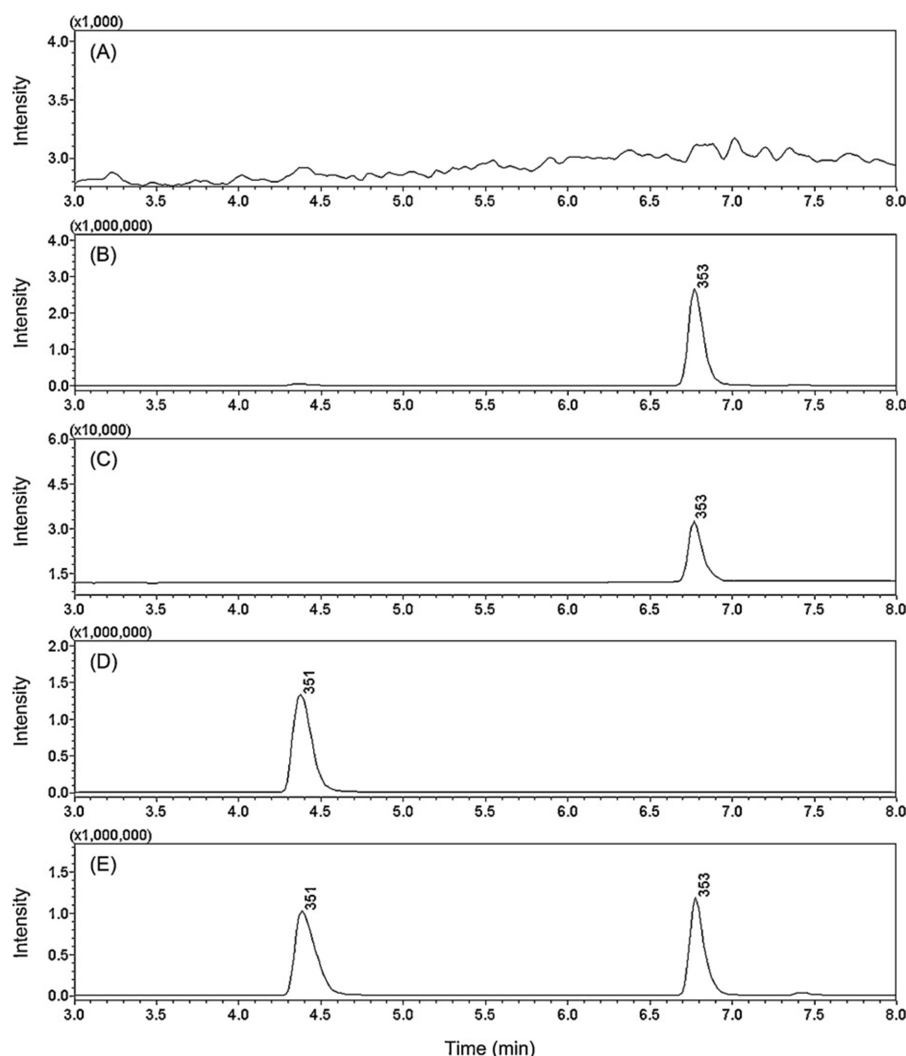

Fig. 2. Selected ion chromatograms of m/z 353 (PG) and m/z 351 (IS) in microsomal sample from HLM: (A) a drug-free blank sample, (B) a blank sample spiked with PG, (C) a blank sample spiked with PG at LLOQ (0.05 μM), (D) a blank sample spiked with IS, and (E) a practice sample after incubation of propofol (100 μM) with HLM (0.5 mg/ml) at 37 °C for 20 min.

For evaluation of the matrix effects, six independent drug-free human hepatic microsomal samples were processed and separately spiked later to obtain QC2, QC3 and QC4. The response (area) was compared with those of the corresponding standard solutions. The matrix effects at these concentrations ranged from 93.5 to 109.2%, while the CVs were less than 5.9%. The recovery exercise was performed at all QC levels ($n = 6$, at each concentration) by comparing the peak area of processed QC samples with those of directly injected QC samples. Mean recoveries for PG at all QC levels were

Table 2
Michaelis–Menten kinetic parameters of propofol *O*-glucuronidation activities in hepatic microsomes from different species.

UGT source	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m (μl/min/mg)
HLM	77.8 ± 7.9	580 ± 11	7.5
CyLM	486 ± 67	1887 ± 89	3.9
PLM	192 ± 28	516 ± 12	2.7
RLM	24.5 ± 3.3	82 ± 2.1	3.4
MLM	55.1 ± 3.2	1736 ± 29	31.5
DLM	N.D.	N.D.	–

N.D. = not detected. The propofol concentration was set at 2–800 μM for HLM, PLM, RLM, MLM and 2–1500 μM for CyLM. Data are expressed as mean ± S.D. ($n = 3$).

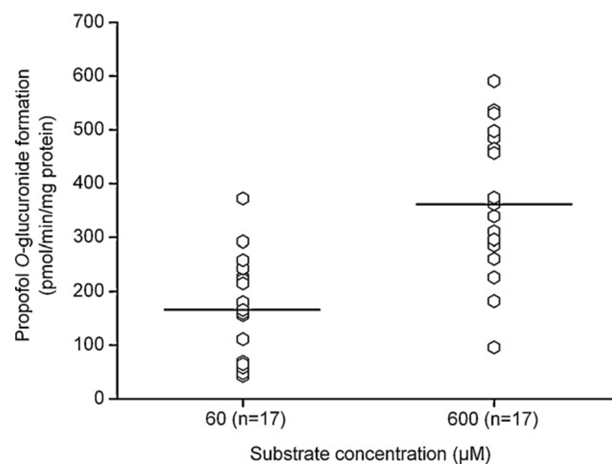

Fig. 3. Comparative rates of PG formation *in vitro* by HLM derived from 17 different donors. All assays were conducted with HLM (0.5 mg/ml) at 37 °C for 20 min. Each point represents the mean of three separate experiments performed in duplicate. Horizontal lines present median values.

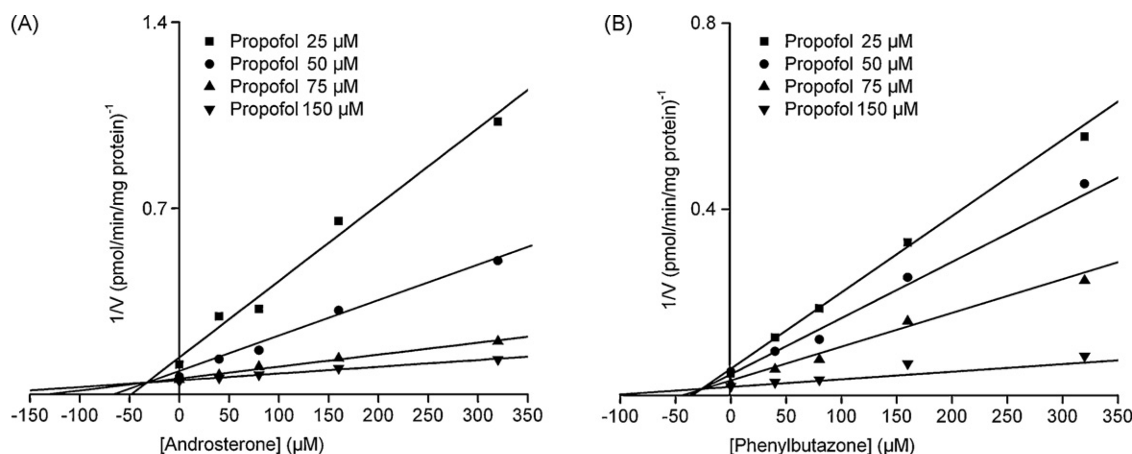

Fig. 4. Dixon plots for androsterone (A) and phenylbutazone (B) inhibition of propofol *O*-glucuronidation using pooled human liver microsomes as enzyme source. All assays were conducted with HLM (0.5 mg/ml) at 37 °C for 20 min. Each point represents the mean of three separate experiments performed in duplicate.

greater than 98.2% with %CV ≤ 6.5, while mean (%CV) recovery of IS (40 μM) was 96.4% (4.7).

Stability experiments were performed using QC samples (three each of QC2, QC3 and QC4) with IS (40 μM) under a variety of storage and process conditions, by comparing peak areas from initial and subsequent determinations. The short-term stability was determined at 25 °C for 0, 4, 12 and 24 h, the results showed that both PG (97.2–102.3%) and IS (≥94.8%) were stable at 25 °C for 24 h. Long-term stability was studied at −20 °C after 0, 4, 12, 24 days of storage, the results showed good stability for both PG (98.1–101.6%) and IS (≥95.4%). The stability of freeze/thaw cycles was also performed over three cycles at −40 °C, satisfying results for PG (93.2–100.5%, %CV) and IS (≥94.1%) were presented. The post-preparative stability was determined the analytes in the UFLC auto-injector at ambient temperature for 0, 4, 12 and 24 h, no significant decomposition was observed for both PG (97.8–102.3%) and IS (≥96.7%). In addition, all assays for PG and IS in stability studies showed an acceptable precision (%CV ≤ 7.9).

Dilution integrity was determined by diluting QC samples (three each of QC3, QC4 or QC5) for 2- and 4-folds with controlled incubation medium. The accuracy for two times diluted samples ranged from 97.2 to 98.4% with a precision (%CV) of 2.6–7.4%, for four times diluted samples ranged from 97.6 to 98.1% with a precision of 2.1–6.9%.

All of these results indicated that the developed UFLC method is specific, sensitive, reproducible and accurate.

3.4. Method application

3.4.1. Kinetic analysis

The formation rates of PG were linear up to 60 min of incubation time (r^2 = 0.997) and up to 1.5 mg/ml of microsomal protein concentrations (r^2 = 0.994) in hepatic microsomes from all species at the propofol concentration of 20 μM. The kinetic determinations were performed using 0.5 mg/ml of microsomal protein concentration (HLM, CyLM, PLM, RLM or MLM) with 20 min incubation, in order to ensure that less than 10% of substrate was biotransformed. Over the tested concentration range, propofol *O*-glucuronidation in HLM, CyLM, PLM, RLM and MLM followed the typical Michaelis–Menten kinetics, as evidenced by the monophasic Eadie–Hofstee plot (data not shown). The kinetic parameters in various hepatic systems were displayed in Table 2. The K_m values in HLM and RLM were comparable with previously published data [12,14,15]. These kinetic parameters can be useful to guide animal selection for the safety/toxicity assessments of propofol, as well as other drugs primarily metabolized by human UGT1A9, considering that glucuronidation (resulting in PG) is responsible for 70% of total propofol clearance in human adults [16].

3.4.2. Human interindividual difference

As shown in Fig. 3, there was 9-fold variation between the highest and lowest activity values (median, 166 pmol/min/mg protein; range, 42.1–372 pmol/min/mg protein) at 60 μM propofol, while 6-fold variation (median, 373 pmol/min/mg protein; range, 94.5–590 pmol/min/mg protein) in enzymatic activity was presented at 600 μM propofol. This finding agreed well with the *in vivo* data reported by Sneyd et al. [16] in which a relevant variability (6–9-fold) in PG versus quinol metabolites (glucuronide and sulphate) in urine from human adults was observed. In addition, more extended interindividual variability in propofol glucuronidation in early human life was also reported [17], which can affect scaling the pharmacokinetics data from rat to children and adults [18]. Recently, the potential toxicity of propofol has drawn much attention [19], which may be attributed by the varied UGT1A9 activities among different individuals. Thus evaluation of the catalytic activity of UGT1A9 in different individuals is very necessary in clinical applications when UGT1A9-mediated glucuronidation plays a major role in clearance of drugs.

3.4.3. Inhibition study

Both androsterone and phenylbutazone showed moderate inhibitory effects on propofol *O*-glucuronidation with IC_{50} values of 98 ± 6.7 and 102 ± 5.4 μM, respectively, which agreed well with a previous report [20]. To further characterize the inhibitory effects of these two inhibitors on the activity of UGT1A9, enzyme-inhibition kinetic experiments were also performed. Dixon plots (Fig. 4) suggested that these two inhibitors competitively inhibited the propofol *O*-glucuronidation activity, with K_i values of 32 ± 2.7 μM for androsterone and 38 ± 3.4 μM for phenylbutazone. These results show that the proposed method is effective and correct for screening of UGT1A9-mediated drug–drug interaction with *in vitro* incubation systems.

4. Conclusion

A UFLC–ESI-MS method was developed and validated for evaluation of propofol *O*-glucuronidation activities in human and animal tissue. The proposed method has shown to be valid, practical and sensitive, suggesting that it is suitable for *in vitro*

studies using PG formation as an index reaction for evaluation of the propofol *O*-glucuronidation activities in different source of enzymes and human UGT1A9-mediated drug–drug interactions.

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