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Biosynthesis and stereoselective analysis of (−)- and (+)-zaltoprofen glucuronide in rat hepatic microsomes and its application to the kinetic analysis

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ABSTRACT

Zaltoprofen, available commercially as a racemic mixture, is a propionic acid derivative of non-steroidal anti-inflammatory drugs (NSAIDs). Firstly, (+)- and (−)-zaltoprofen glucuronide was biosynthesized and purified. Then a simple and rapid RP-HPLC analysis method for direct determination of (+)- and (−)-zaltoprofen glucuronide in rat hepatic microsomes was developed and validated. The calibration curves of (+)- and (−)-zaltoprofen glucuronide both showed good linearity in the concentration range from 0.15 to 31.13 μM. The lower limit of quantification was 0.15 μM. Finally, this method was used to investigate the enantioselectivity of zaltoprofen glucuronidation in rat hepatic microsomes. The kinetics of zaltoprofen glucuronidation in rat hepatic microsomes for 40 min incubation fit the Michaelis–Menten model. Kinetic analysis indicated that (−)-zaltoprofen had a higher glucuronidation rate in rat liver microsome than that of (+)-zaltoprofen. The catalyzing efficiency (V_{max}/K_m) ratio of (+)-zaltoprofen to (−)-enantiomer is 0.8 times in rat liver microsomes.

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1. Introduction

Zaltoprofen (ZPF), 2-(10,11-dihydro-10-oxodibenzo(*b,f*)thiepin-2-yl) propionic acid (Fig. 1A), is a derivative of 2-arylpropionic acids (2-APA), an important group of nonsteroidal anti-inflammatory drugs (NSAIDs) and has powerful inhibitory effects on acute and chronic inflammation with less adverse reactions on the gastrointestinal tract than other NSAIDs [1]. ZPF exerts anti-inflammatory actions and analgesic effects by inhibiting prostaglandin synthesis and through a peripheral mechanism by inhibition of bradykinin B_2 receptor-mediated bradykinin responses in primary afferent neurons [2–4]. Bawolak et al. [5] found that ZPF potently suppressed the relaxant response recruited by the kinin, which implied that ZPF was more potent than ibuprofen in this respect. ZPF was already used in the treatment of rheumatoid arthritis and osteoarthritis as well as to relieve inflammation and pain after surgery, injury and tooth extraction. Recently, a double-blind study [6] conducted in 170 patients indicated that the effect and safety of ZPF as a single dose to reduce inflammation in acute upper respiratory tract infection was also excellent, which provided a scientific evidence on the proper use of ZPF in the therapy of acute respiratory infections.

The 2-APAs contain at least one chiral center and exhibit optical activity. In general, enantiomers have similar physicochemical properties, but they may show quite different pharmacological and pharmaceutical properties. ZPF, available commercially as a racemic mixture, has one chiral center at the alpha carbon to the carboxylic acid. It is predominantly metabolized by CYP2C9 and UGT2B7, and is excreted to the urine as zaltoprofen-1-O-acyl-glucuronide [7], which is formed catalytically by UDP-glucuronosyltransferases (UGTs). The glucuronidation involves the transfer of the glucuronic acid moiety of uridine 5-diphosphoglucuronic acid (UDPGA) to the substrate, rendering the drug polarity thus more readily excreted in urine. According to the research by Furuta et al. [7], ZPF might be metabolized mainly by UGT2B7 rather than by CYP isoforms, which meant that the glucuronidation was a very important metabolizing pathway for ZPF. Stereoselective glucuronidation has been reported for other profen NSAIDs. Racemic Flurbiprofen was stereoselectively glucuronidated by recombinant UGT isozymes and UGT2B7 formed the (R)-glucuronide at a rate 2.8-fold higher than that for (S)-glucuronide [8]. Gauthier et al. [9] reported that the glucuronidation of carprofen was stereoselective and species-dependent and the rat exhibited a high stereoselective glucuronidation as compared with other species. The ratio of the enzymatic efficacies of R/S carprofen in rat hepatic microsomes when incubations were performed in the presence of the racemic

Abbreviations: LOD, limit of detection; LOQ, limit of quantitation; RP-HPLC, reverse phase high performance liquid chromatography; UGT, uridine 5-diphosphoglucuronosyl transferases; UDPGA, uridine 5-diphosphoglucuronic acid.

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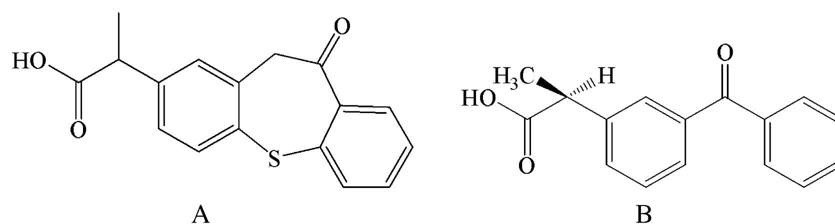

Fig. 1. Chemical structures of (A) ZPF and (B) S-(+)-ketoprofen (IS).

carprofen and enantiomeric carprofen was 4.2 and 3.8 individually, which indicated that the R-enantiomer was glucuronidated at a more efficient rate than its enantiomorph. However, the stereoselectivity of the glucuronidation between the enantiomers of ZPF was not yet studied.

Although earlier publications have described column-switching high-performance liquid chromatography/ultraviolet (HPLC-UV) method [10,11] and a liquid chromatography with electrospray tandem mass spectrometry method [12] for the analysis of zaltoprofen in biological samples, at present, there are few reports about the direct determination of zaltoprofen glucuronide formed by *in vitro* or *in vivo* assay since it is very difficult to obtain the standards of the glucuronides. Previously reported the glucuronidation of ZPF was measured through the disappearance of ZPF or as patent drug equivalents after enzymatic hydrolysis [7], which was obviously lack of precise and very tedious. Therefore, one of the purposes in this study was to develop a simple HPLC-UV method for directly determining zaltoprofen glucuronide (ZPFG) which can be employed to investigate the enantioselectivity of ZPF glucuronidation metabolism. Since the (+)-ZPF and (−)-ZPF were unavailable, we prepared and purified enantiomeric ZPF on preparative HPLC firstly. The glucuronide reference standards of (−)- and (+)-ZPF enantiomers were biosynthesized and purified using solid phase extraction.

According to the literatures, no kinetic analysis of ZPF glucuronidation in rat liver microsomes has been performed. It is still unclear whether the glucuronidation is preferred by one of enantiomers. Thus the second purpose of the present study was to thoroughly characterize ZPF glucuronidation in rat liver microsomes.

2. Experimental

2.1. Chemicals and reagents

Rac-ZPF (chemical purity >99.0%) was obtained from Yikang Pharm. Co. Ltd. (Tengzhou, Shandong, China). (+)-ZPF and (−)-ZPF were obtained by preparative HPLC, S-(+)-ketoprofen, uridine 5-diphosphoglucuronic acid (UDPGA), phenobarbital, alamethicin and β-D-glucuronidase were purchased from Sigma Chemical Co. (St Louis, MO, USA). The SampliQ C18 cartridges (500 mg, 3 mL) were purchased from Agilent Technologies (Palo Alto, CA). Chloroform, anhydrous sodium sulfate, trifluoroacetic acid (TFA), ammonium acetate, acetic acid, formic acid, hexane, isopropyl alcohol, acetonitrile, $MgCl_2$, $CaCl_2$, NaCl, Tris–HCl and other chemicals and solvents were analytical reagents or chromatographic grade and obtained from common commercial sources.

The rat hepatic microsomes were prepared according to the method of Zeng et al. [13] from the rats (Sprague-Dawley, male, 190–220 g, were obtained from the Lab Animal Center of Shandong University), which were treated with phenobarbital (i.p. 80 mg/kg, 3 days). They were raised in a room maintained at $22 \pm 2\,^{\circ}C$ with relative humidity of $50 \pm 10\%$ and had free access to water and food prior to experiment. Microsomal protein was measured by the method of Lowry et al. with bovine serum albumin as the standard protein [14]. The study was approved by the Animal Ethics Committee of Shandong University.

2.2. Preparation of (+)- and (−)-ZPF isomer

Preparative HPLC was performed on a YMC K-Prep HPLC instrument (YMC Co., Ltd. Kyoto, Japan) consisting of a Soma UV detector S-3120 (Soma Optics, Ltd. Kyoto, Japan) with a mobile phase consisting of hexane, isopropyl alcohol and acetic acid (90:10:0.1, v/v/v). The preparative column was a Chiralcel OJ-H, 25 cm × 2 cm I.D. (Daicel, Tokyo, Japan). The HPLC system used for chiral purity testing of enantiomeric ZPF consisted of an LC 20AT pump (Shimadzu, Kyoto, Japan) and SPD-20A UV detector (Shimadzu, Kyoto, Japan). The mobile phase was a mixture of hexane, isopropyl alcohol and acetic acid (90:10:0.1, v/v/v). The chiral purity determination was carried out using Chiralcel OJ-H (150 mm × 4.6 mm I.D.) column (Daicel, Tokyo, Japan). The specific rotation of (+)- and (−)-ZPF was performed on Gyromat-HP Digital Automatic Polarimeter (Kernchen, Germany).

2.3. Biosynthesis and purification of ZPFG

(−)- and (+)-ZPFGs were biosynthesized using (−)- or (+)-ZPF as the substrate. The incubation mixture (total volume of 100 mL) contained 100 mM Tris–HCl buffer (pH 7.4), 10 mM $MgCl_2$, 5 mM UDP-glucuronic acid (UDPGA), 15 μg/mL alamethicin, 100 μM ZPF, and 1 mg/mL microsomal protein. ZPF was dissolved in DMSO. After pre-incubation at 37 °C for 5 min, UDPGA was added as cofactor to initiate the reaction. The incubation concentration of ZPF was 100 μM. The two-fold volume of $CHCl_3$ and 4 mL of 10% formic acid were added after 2 h incubation. After the mixture was vortexed, the protein was precipitated by centrifugation and the remaining ZPF was extracted with $CHCl_3$. The aqueous fraction was transferred into a clean tube and the two-fold volume of $CHCl_3$ was added again. The procedure was repeated to completely detach the remaining ZPF, which was confirmed with HPLC. The aqueous layer after extraction was loaded onto Agilent SampliQ C18 cartridges (500 mg, 3 mL). Each cartridge was then washed with 2 mL of water-TFA (90:10). Glucuronides were eluted with 2 mL of acetonitrile. The final elution solution was evaporated to dryness under N_2 at 37 °C, and the residue was reconstituted with 1000 μL deionized water which was then used as the standard stock solution for the assay of ZPFG.

2.4. LC–MS analysis of ZPFG

The incubation of ZPF with rat liver microsomes for structure identification was carried out as described in Section 2.3. Detection of ZPFG was achieved with injection of 10 μL of the centrifugal supernatant onto the HPLC–MS system. HPLC–MS analysis was performed on an HPLC/electrospray ionization (ESI)–MS/MS system that included an ACCELA HPLC system coupled with an LTQ-Orbitrap XL mass spectrometer with an ESI source (ThermoFinnigan, Bremen, Germany). The precolumn (C18, 2 μm) and analytical column (C18, 4.6 mm × 150 mm, 5 μm) were purchased

from Phenomenex (Torrance, CA, USA). Data was collected and analyzed by the Xcalibur Qual Browser software (version 2.0.3, Thermo Fisher Scientific, San Jose, CA, USA).

Ionization of the glucuronides was achieved by electrospray in the positive ion mode. The HPLC separation was carried out at 20 °C using a mobile phase consisted of 10 mM, pH 5.0 ammonium acetate in water (solution A) and acetonitrile (solution B). The solvent program consisted of an initial isocratic mobile phase mix (27% solution B) for 15 min, followed by a linear gradient from 27% to 36% solution B over 1 min. The mobile phase flow rate was 1.0 mL/min with a 2:3 split. The ionization voltage was 4.5 kV and the capillary temperature was set at 300 °C. Nitrogen was used as both the sheath and auxiliary gas at flow rate of 50 arb and 10 arb respectively, 50 arb; auxiliary gas, 10 arb; The capillary potential was 25 V.

The structures of the two glucuronides were identified by LC–MS operating in the ESI mode.

2.5. Quantification of ZPFG by hydrolysis with β-glucuronidase

A 4 μL aliquot of the ZPFG stock solution and 4 μL of the β-glucuronidase solution (20 U/μL) were added into 192 μL of PBS (pH 5.0) [15]. After incubation at 37 °C for 24 h, 40 μL of (+)-ketoprofen solution (80 μM, dissolved in DMSO as the internal standard, IS) and 160 μL of acetonitrile were added to into the solution. Triplicate samples and three control samples without β-glucuronidase were examined. The mixture was vortexed for 3 min and centrifuged at 13,000 rpm for 10 min. 20 μL of the supernatant was injected into HPLC system. The ZPFG was completely hydrolyzed to ZPF and the content of ZPF was analyzed accurately by HPLC method described herein. The peak area ratio of ZPF in hydrolytes versus IS was compared using the calibration curve prepared by ZPF.

2.6. ZPF glucuronidation assay

The reaction mixture was incubated at 37 °C for 40 min as previously described in Section 2.3 and the total volume of incubate was 100 μL. The enzymatic reaction was terminated by adding 30 μL of ice-cold acetonitrile and 10 μL of 10% formic acid at the designated time at 37 °C in a shaking water bath. A 20 μL aliquot of 80 μM IS was added. After removal of the protein by centrifugation at 13,000 rpm for 10 min. A 20 μL portion of the supernatant was subjected to HPLC.

HPLC–UV analyses of ZPFG and ZPF were performed on an Agilent HPLC system (model 1200; Agilent Technologies, Palo Alto, CA) consisting of autoinjector, binary pump, UV absorbance detector set at a wavelength of 241 nm. The column and the mobile phase were the same as described in Section 2.4. Metabolite concentrations in the incubation were determined using a standard curve of peak area ratios (normalized to the internal standard) to the concentrations.

2.7. Preparation of calibration standards and quality control samples

Stock solutions of Rac-ZPF (5 mM) and IS (0.4 mM) were prepared in DMSO. Working solutions of ZPF in the concentration range from 0.5 to 200 μM and IS (80 μM) were prepared by diluting the stock solution with PBS (pH 5.0). Quality control (QC) working solutions of ZPF (1.78, 97.9, 195.8 μM) were similarly prepared. The mixture was vortexed for 3 min and centrifuged at 13,000 rpm for 10 min. 20 μL of the supernatant was injected into HPLC system.

The calibration standards of ZPFG in the concentration range from 0.15 μM to 31.13 μM were prepared by spiking a series of stock solution of (+)- and (−)-ZPFG with blank microsomes incubates respectively. QC working solutions of ZPFGs (0.31, 15.56, 24.90 μM) were similarly prepared. The mixture was mixed with 10 μL of 10% formic acid, 30 μL of acetonitrile and 20 μL of 80 μM (+)-ketoprofen. After removal of the protein by centrifugation at 13,000 rpm for 10 min, a 20 μL aliquot of supernatant was injected into HPLC for analysis.

2.8. Method validation

The method was validated through linearity, intra-day and inter-day precision, accuracy and stability. Assay accuracy and precision were assessed by determining QC samples at four concentration levels. The accuracy was determined by comparing the calculated concentration to the theoretical concentration of the QC samples. A series of hydrolysis solutions, spiked with various amounts of ZPF, were processed according to the procedure previously described in Section 2.7. The peak area ratios of ZPF to the IS were compared with the calibration curve of ZPF. Another series of blank microsomes incubates, added with (+)-and (−)-ZPFG at different concentrations, were processed as previously described in Section 2.7. The peak area ratios of ZPFGs versus the IS were compared with the calibration curve of (+)- and (−)-ZPFG, respectively. For these two series of assays, intra-day variability was determined by analyzing five parallel samples, and inter-day variability was determined by analyzing samples on five separate days.

The stability of ZPFG in rat microsomes was evaluated using QC samples of ZPFG at three concentrations (0.31, 15.56, 24.90 μM) with five samples for each concentration. The stability of ZPFG was tested under the following conditions: (1) freeze–thaw stability of ZPFG in rat microsomes through three freeze–thaw cycle. QC samples at three different concentrations were thawed at room temperature and refrozen at −20 °C over three cycles and assayed; (2) short-term stability of ZPFG in rat microsomes at room temperature for 12 h; (3) incubated stability of ZPFG in rat microsomes at 37 °C water bath for 12 h. All the QC samples for stability assessment were analyzed in quintuple.

2.9. Kinetic analysis

The studies were approved by the Animal Ethics Committee of Shandong University. Kinetic analysis of ZPF glucuronidation was performed in rat liver microsomes obtained from experimental animals ($n = 5$). Rac-ZPF was added into 0.1 mL incubation solution described previously. For the time-course experiment, the samples were incubated at one concentration of ZPF at 37 °C for 10, 20, 30, 40, 60, 80, and 100 min. For the dose-course experiment, the samples were incubated for 40 min at a series of concentrations ranging from 9.79 to 1099.3 μM. The concentration of microsomal protein was 1 mg/mL, and incubation time was 40 min. The concentration of UDPGA in the incubation mixture was 5 mM. Three replicate samples were examined for every time point and each concentration. The metabolite was detected and quantified according to the calibration curve of ZPFG. Kinetic parameters were estimated from the fitted curves using the GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA), designed for nonlinear regression analysis. The following equation was applied, assuming a Michaelis–Menten equation: $V = V_{max} \times [S]/(K_m + [S])$, where V is the rate of reaction, V_{max} is the maximum velocity, K_m is the substrate concentration at which the rate of metabolism is 50% of V_{max}, and $[S]$ is the substrate concentration.

3. Results and discussion

3.1. Purity determination of (+)-ZPF and (−)-ZPF

The enantiomeric excesses for both enantiomers were all more than 98.0% determined by HPLC method described in Section

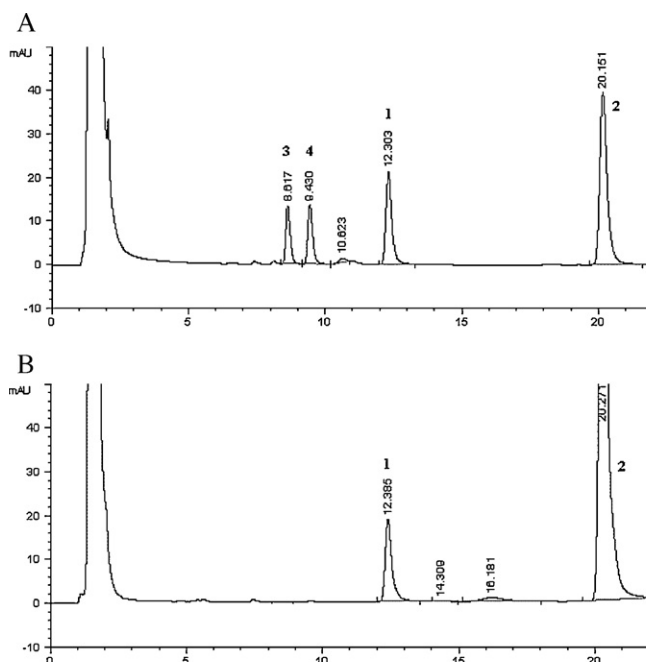

Fig. 2. Representative chromatograms of HPLC analysis of the (+)- and (−)-ZPFG formation from racemic-ZPF in rat liver microsomes. Pooled rat liver microsomes (1 mg/mL) were incubated with racemic ZPF and with (A) and without (B) 5 mM UDPGA at 37 °C for 40 min. Peak 1: S-(+)-ketoprofen; Peak 2: (±)-ZPF; Peak 3: (+)-ZPFG; Peak 4: (−)-ZPFG. HPLC conditions are given in Section 2.4.

2.2. The specific rotation of (+)- and (−)-ZPF was $[\alpha]_D^{20} = 33.332$ (3.01 mg/mL) and $[\alpha]_D^{20} = -32.921$ (2.83 mg/mL) individually. Comparing with the method reported in literature [16], which was used to determine the enantiomeric purity of ZPF, the method we used may be more suitable for the chiral preparation of isomeric ZPF because of the application of volatile mobile phases.

3.2. Characterization of ZPFG by RP-HPLC and LC–MS

Chromatographic separation of (+)- and (−)-ZPFG, (+)-ketoprofen and (±)-ZPF was satisfactory, with no interfering peaks. Typical chromatograms of the incubation mixture are shown in Fig. 2. Chromatograms of control samples, including the incubation mixture without microsomes or substrate, also indicated that there were no interfering peaks (data not shown).

The LTQ-Orbitrap XL mass spectrums of the peaks formed by incubation of (±)-ZPF with rat liver microsomes is shown in Fig. 3. The $[M+NH_4]^+$ peak at m/z 492.1 and the $[M+H]^+$ ionic peak at m/z 475.1 corresponded to ZPFG. A fragment ion at m/z 299.1 corresponded to the parent drug ZPF+H.

3.3. Calibration curve of ZPF

The calibration curve of ZPF was constructed by plotting the peak area ratios (y) of the ZPF to the IS against the concentration (x) of ZPF. It was found that ZPF concentrations were linearly related to ZPF versus the IS area ratios over the range investigated. The regression equation of the calibration curve of ZPF was $y = 0.0054x - 0.0343$ ($r^2 = 0.9993$, $n = 5$). The lower limit of quantification (LLOQ) of ZPF was established at 0.5 μM. The LLOQ was accepted with a relative standard deviation of less than 10% and 92% accuracy for the analysis for ZPF.

3.4. Quantification of (+)- and (−)-ZPFG by hydrolysis

ZPFGs were hydrolyzed thoroughly by β-glucuronidase after 24 h incubation. According to the calibration curve of ZPF, the area ratios of ZPF after hydrolysis versus the IS were used to calculate the concentration of the ZPF transformed from ZPFG by hydrolysis. The concentration of the stock (+)- and (−)-ZPFG solution were calculated as being 116.31 μM and 210.56 μM, respectively.

3.5. Calibration curves of ZPFGs

The calibration curves of ZPFGs were constructed by plotting the peak area ratios (y) of the (+)- and (−)-ZPFG to the IS against the concentration (x) of (+)- and (−)-ZPFG. The regression equations of the calibration curves of (+)- and (−)-ZPFG were $y = 0.0288x + 0.0044$ ($r^2 = 0.9993$, $n = 5$) and $y = 0.0377x - 0.0102$ ($r^2 = 0.9995$, $n = 5$), respectively. The possible reason that causes the difference between the slopes of the two calibration curves is due to the different elution order of the two glucuronides in the chromatographic separation. Thus, the peak shapes of the two glucuronides are different, which may lead to different integration results. The similar phenomenon had also been reported by Yu et al. [15]. The LLOQ of ZPFG was established at 0.15 μM. The LLOQ was accepted with a relative standard deviation of less than 12% and 93–104% accuracy for the analysis for ZPFG.

3.6. Accuracy and precision

The results of the accuracy and inter- and intra-day precision for the assay of ZPF at four concentrations of ZPF are listed in Table 1. The results of the accuracy and inter- and intra-day precision for the assay of ZPFG are shown in Table 2. The methods were found to be highly precise with intra-day precision <10.3% and inter-day precision <11.6% at each concentration of QC and LLOQ sample tested. The accuracy of ZPF was in the range of 92.0–103.9%. The accuracies of (−)- and (+)-ZPFG in rat hepatic microsomes were in the range of 92.4–103.7% and 93.9–104.1%, respectively.

3.7. Stability of ZPFG

QC samples of ZPFG at three concentrations were used for stability experiments. The stability of ZPFG was tested under different conditions. The results (Table 3) indicated that ZPFG was stable under routine laboratory conditions.

We investigated the validation of this assay method. These results indicate that the proposed method is satisfactory for the direct determination of the diastereomeric zaltoprofen glucuronides in the rat hepatic microsomes with high accuracy and precision.

Table 1
Accuracy and precision for assay of ZPF (mean ± SD, $n = 5$).

Theoretical concentration (μM)	Accuracy (%)	Intra-day variability RSD (%)	Inter-day variability RSD (%)
0.5	92.0 ± 9.7	10.1	9.0
1.78	103.9 ± 6.9	8.3	7.3
97.9	96.3 ± 7.4	4.1	5.5
195.8	97.1 ± 4.3	3.8	4.7

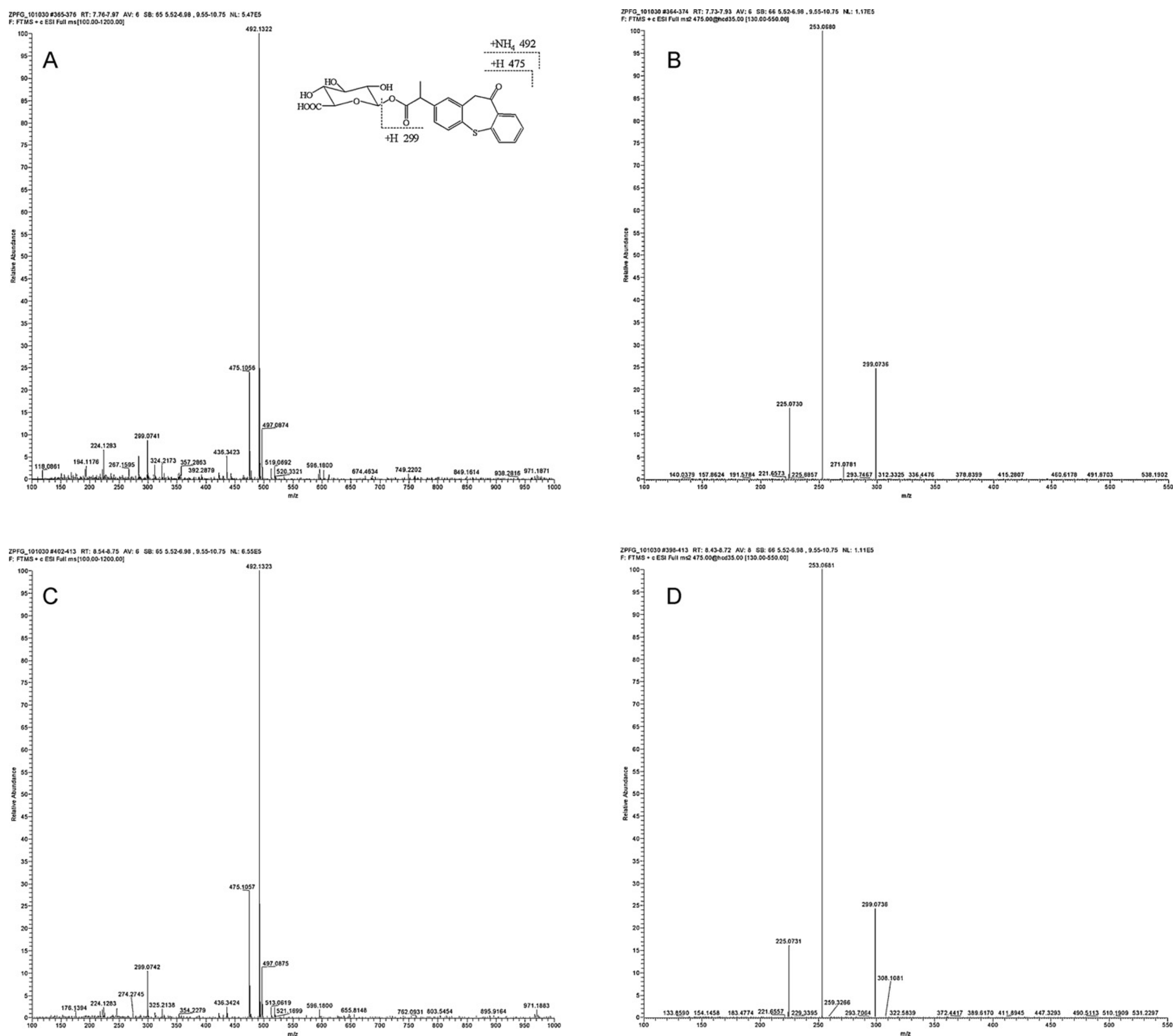

Fig. 3. Representative MS and MS/MS spectra of protonated (+)-ZPFG (A) (B) and (−)-ZPFG (C) (D) formed from ZPF by rat liver microsomes.

Meanwhile, previous studies have shown that the acyl glucuronides are chemically very active and react with amino groups on proteins to produce protein-bound adducts [17,18], which may be responsible for the hypersensitivity reactions of acidic compounds [19,20]. It is also reported that rat hepatic bile acid acyl glucuronosyltransferase is effectively inhibited by the amino acid conjugates of bile acids, which may be important as a detoxification system in the body [21]. Acyl glucuronides have been hypothesized to be associated with toxicity. Therefore, the developed method would be also very useful for the characterization of hepatic acyl glucuronosyltransferase for acidic drugs.

3.8. Kinetics of ZPF glucuronidation in rat liver microsomes

The profens have a chiral center within their structures that exhibit optical activity and contain a pair of stereoisomers. One of

Table 2
Accuracy and precision for assay of (−)- and (+)-ZPFG in microsomal incubates (mean ± SD, $n = 5$).

Concentration spiked (μM)	Accuracy (%)		Average relative standard deviation (%)			
			Intra-day		Inter-day	
	(−)	(+)	(−)	(+)	(−)	(+)
0.15	103.7 ± 11.1	93.9 ± 9.8	9.9	10.3	11.6	10.2
0.31	92.4 ± 7.6	95.7 ± 8.0	8.9	7.5	9.9	8.3
15.56	102.9 ± 5.3	104.1 ± 4.8	5.1	4.6	5.7	7.0
24.90	101.7 ± 2.0	103.9 ± 1.8	3.2	2.8	2.2	1.4

Table 3
The stability of ZPFG in rat microsomes under different conditions (mean ± SD, $n = 5$).

Concentration (μM)	Percentage of initial value	
	mean ± SD	RSD (%)
Freeze–thaw stability (−20 °C/room temperature, 3 cycles)		
0.31	95.9 ± 2.1	2.2
15.56	98.1 ± 3.0	3.1
24.90	102.3 ± 2.5	2.4
Short-term stability (room temperature for 12 h)		
0.31	106.4 ± 3.8	3.6
15.56	97.9 ± 3.0	3.1
24.90	101.9 ± 2.7	2.6
Incubated stability (37 °C for 12 h)		
0.31	97.8 ± 4.8	4.9
15.56	103.5 ± 3.9	3.8
24.90	96.5 ± 1.1	1.1

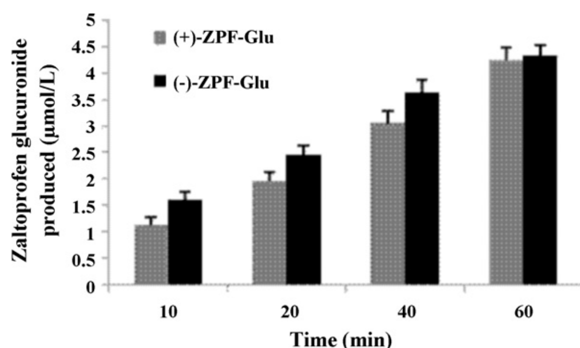

Fig. 4. Time course for racemic ZPF (97.9 μM for each enantiomer) incubated with rat liver microsomes.

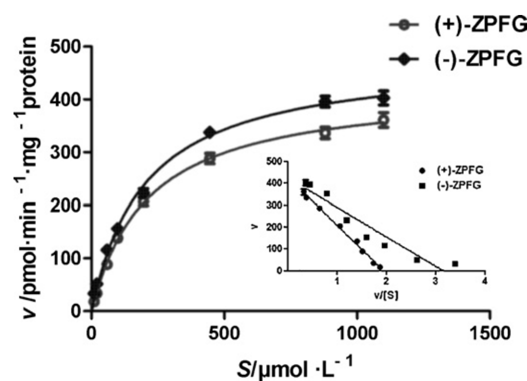

Fig. 5. Kinetics of racemic ZPF glucuronidation in rat liver microsomes. The concentration of ZPF ranged from 9.79 to 1099.3 μM. The formation of ZPFG was determined as described in Section 2. The inset shows the Eadie–Hofstee plot of the experimental data. Each incubation was performed by triplicate determinations.

the primary pathways of the profens metabolism is glucuronidation, in which a polar sugar is conjugated to the profens, increasing its rate of elimination from the body [22]. It is well known that their stereoisomers might show different behaviors in terms of chiral inversion and glucuronidation among enantiomers [23–27]. (+)- and (−)-ZPFGs were produced linearly over the time range studied (shown in Fig. 4). In our studies, it was found interestingly that the production of (−)-ZPFG was higher than that of (+)-ZPFG within the first 60 min, but with the incubation time extending, the difference in production decreased gradually. After being incubated for 80 min, the production of (+)-ZPFG was higher than that of (−)-ZPFG inversely (data not shown). It was found that (+)-ZPFG was almost 1.5 fold of (−)-ZPFG after 100 min incubation. Initially, it was suspected that this phenomenon was produced because of the instability of ZPFG in the incubation mixture. However, the stability experiments (see Table 3) had proved that ZPFG was very stable in the incubation mixture. Previously studies indicated that the effect on cyclo-oxygenase of fenoprofen and ketoprofen were resulted from the S-(+) enantiomer [28,29]. Among them, the metabolic chiral inversion process corresponds to a selective unidirectional transformation from the inactive R-(−) to the active S-(+) enantiomer [30,31]. Therefore, we may presume that with the incubation time expanding, the possible metabolic chiral transformation inverses and exacerbates the stereoselective glucuronidation of ZPF in rat liver microsomes.

As shown in Fig. 5, the ZPF glucuronidation by rat liver microsomes displayed Michaelis–Menten kinetics (all of $R^2 > 0.99$). The Eadie–Hofstee plot was monophasic. The kinetic parameters were shown in Table 4. No differences found in the values of K_m indicated that (+)- and (−)-ZPF had similar affinity for rat liver microsomes. The glucuronidation rate of (+)-ZPF and (−)-ZPF catalyzed by rat liver microsomes was significantly different ($P < 0.01$). And the metabolic activity (V_{max}/K_m) of (+)-ZPFG to (−)-ZPFG by rat liver microsomes is about 0.8 times for racemic ZPF under the determined conditions. According to the previous studies, many NSAIDs demonstrated stereoselective metabolism and kinetic disposition in rat, including ketoprofen [32], fenoprofen [33], pranoprofen [34], ibuprofen [35] and flurbiprofen [36]. Acyl glucuronides (AGs), however, have electrophilic reactivity which can lead to covalent binding with protein [37,38]. One possible mechanism for the formation of these adducts involves the spontaneous migration of the acyl group to the 2-, 3-, or 4-hydroxyl groups of the sugar moiety, tautomerization of the pyranose ring to its aldose form, and condensation of the aldehyde group of the ring-opened tautomer with a lysine €-amino on the protein to form an amide [38]. The produced covalent protein adducts increase the risk of idiosyncratic drug toxicity (IDT) including a wide range of adverse drug effects such as drug hypersensitivity reactions and cellular toxicity [39]. As a result of this metabolic pathway and its biological consequences, a number of nonsteroidal anti-inflammatory drugs cause adverse reactions in humans, as exemplified by benoxaprofen [40]. In the case of UGTs being induced or in kidney disease, the toxic glucuronides will accumulate and this will increase the risk of IDT. It is essential that candidate drugs have low risk of IDT [41]. Glucuronidation of the two enantiomers at different reaction rates may lead to varying profiles of efficacy and toxicity [42]. It is necessary to elucidate the differences in toxicology, pharmacology and

Table 4
Enzymatic kinetic parameters of ZPF with rat liver microsomes (mean ± SD, $n = 3$).

Zaltoprofen	K_m (μM)	V_{max} (pmol/min/mg protein)	V_{max}/K_m (μL/min/mg protein)	(+)/(−)
(+)	210.1 ± 14.3	424.5 ± 9.6[a]	2.02 ± 0.15[b]	0.8
(−)	191.2 ± 13.3	481.5 ± 10.7	2.52 ± 0.14	

Rat liver microsomes were incubated for 40 min at 37 °C in the presence of 5 mM UDPGA and increasing concentration of rac-ZPF (9.79–1099.3 μM) in 100 mM Tris–HCl buffer (pH 7.4). Values are kinetic constant ± SD determined in triplicate in rat microsomes provided from five animals. (+)/(−) Ratio of the enzymatic efficacies of (+)-ZPF to (−)-ZPF.

[a] Values significantly different from the corresponding value of the enantiomorph ($P < 0.01$).
[b] Values significantly different from the corresponding value of the enantiomorph ($P < 0.05$).

pharmacokinetics between the two enantiomers to provide a drug that meets the requirement of low IDT.

For zaltoprofen, it was firstly found that the V_{max} value of (−)-ZPF glucuronidation in rat hepatic liver microsomes was significantly higher than that of (+)-ZPF enantiomer in the present study. However, due to the possibly existed chiral transformation, further experiments would be necessary to elucidate the stereoselective metabolism of racemic ZPF.

4. Conclusions

In this study, a simple, sensitive and robust RP-HPLC method was established to simultaneously determine (+)- and (−)-ZPFG in rat liver microsomes. And the method was applied to study the stereoselectivity of ZPF glucuronidation in rat hepatic microsomes. The results showed that there are stereoselective differences of kinetics in rat hepatic microsomes between the two enantiomers.

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