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Quantitation of celecoxib and four of its metabolites in rat blood by UPLC-MS/MS clarifies their blood distribution patterns and provides more accurate pharmacokinetics profiles

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Abstract

A sensitive UPLC-MS/MS method was established and validated for the quantitation of celecoxib and its metabolites in rat blood. The analytes were extracted from rat blood samples by a salting-out liquid-liquid extraction method followed by the UPLC chromatography. The mass analysis of effluent was performed on an API 5500 Qtrap mass spectrometer via multiple reactions monitoring (MRM). The linear response ranges were 0.3–20000 nM for celecoxib, and 1.2–20000 nM, 0.3–20000 nM, 2.0–2000 nM, 1.5–6000 nM for its metabolites carboxycelecoxib (M2), hydroxycelecoxib (M3), hydroxycelecoxib glucuronide (M1), and carboxycelecoxib glucuronide (M5), respectively. The inter-day and intra-day accuracies were within 85–115%, and the inter-day and intra-day precision were acceptable (<12%) for all analytes. Recoveries were above 70% and no obvious matrix effects were observed. The validated UPLC-MS/MS method was successfully applied to a pharmacokinetics study of oral celecoxib (20mg/kg) in Sprague-Dawley rats, and the rat blood concentrations (0–48 hr) of celecoxib and two of its metabolites M2 and M3 were successfully determined. Using the same method, we also showed preferential distributions of celecoxib, M2 and M3 in the blood cells as compared to the plasma. In conclusion, our results showed that our validated LC-MS/MS method can be successfully used for the pharmacokinetic studies of celecoxib and that the blood cells are a very important compartment for this drug such that profiles of celecoxib and its metabolites in whole blood will be more comprehensive and accurate representation of their profiles in vivo than the plasma.

Keywords

Celecoxib; metabolites; pharmacokinetics; UPLC-MS/MS; drug distribution

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1. Introduction

Cyclooxygenases (COXs) are a class of enzymes which convert arachidonic acid to prostanoids [1-3], and two COX isoforms have been identified in human: COX-1 and COX-2 [4]. COX-1 is constitutively expressed in many tissues, and its activity is essential in maintaining the functions of platelets, gastrointestinal (GI) tract, and kidney. [5]. In contrast, COX-2 is an inducible enzyme overexpressed as the result of inflammation and/or carcinogenesis. Since the late 1980s', COX-2 has been extensively investigated as a therapeutic target for the treatment of inflammation and certain types of cancer [6-8]. The traditional non-steroid anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin usually work as dual inhibitors of both COX-1 and COX-2 [9]. However the administration of conventional NSAIDs in patients may lead to serious side effects in the GI tract, because the mucosal integrity is compromised when COX-1 is inhibited [10]. The COX-2 selective inhibitors were new NSAIDs with minimal or much less severe side effects because they do not inhibit the physiological functions of COX-1 [11,12]. Celecoxib (Figure 1) and rofecoxib, the two most important COX-2 selective inhibitors, were launched in the market at the end of last century and soon became blockbuster drugs for the treatment of osteoarthritis (OA) and rheumatoid arthritis (RA). Today, only celecoxib is on the market whereas rofecoxib was withdrawn from the market.

The Cox-2 protein also plays an important role in the carcinogenesis of several types of cancer [13-15]. In the clinical trials determining the efficacy of celecoxib and rofecoxib in the prevention of colorectal cancer, these two drugs significantly reduced the occurrence of colorectal adenoma in high-risk patients [16,17]. Although routine and long-term administration of the selective COX-2 inhibitors may be associated with an increased risk of the cardiovascular events, celecoxib was under active investigation for its application as a promising agent in the chemoprevention of colorectal cancer, breast cancer, lung cancer, prostate cancer and etc. [18-21].

In humans and rats, the metabolism pathways of celecoxib are similar. In previous studies, orally administered celecoxib was demonstrated to be well absorbed and appeared in the plasma quickly, featuring a concentration much higher than its IC_{50} value against COX-2 [22]. The phase I metabolism of celecoxib comprises of two steps (Figure 1, A) [23,24], where celecoxib is initially oxidized to hydroxycelecoxib (M3), and then further oxidized to carboxycelecoxib (M2). In humans, these two steps are catalyzed by CYP2C9 and alcohol dehydrogenase, respectively [25,26]. In subsequent phase II metabolism, M3 and M2 can be conjugated with glucuronic acid on the carboxyl or hydroxyl group to form the glucuronides M1 and M5, respectively. The uridine 5'-diphospho-glucuronosyltransferases (UGTs) are responsible for the glucuronidation. It is also notable that as 1-*O*-acyl glucuronide, M1 is unstable at physiological pH and isomers can be formed via the acyl migration process (Figure 1, C) [27]. M1 can be converted to M2 by β -glucuronidase, while the acyl migration products are resistant to hydrolysis [28]. In previous studies, multiple positional isomers of M1 were observed in the in vivo samples [24]. All these metabolites of celecoxib were inactive in inhibiting COX-2 activity [29].

In the early studies, the investigation into the absorption, disposition, metabolism and excretion of celecoxib were conducted using ^{14}C -labeled celecoxib [23,24]. Later, several analytical methods employing high performance liquid chromatography (HPLC) and ultraviolet (UV) absorbance detector were established for celecoxib [30-32]. Mass spectrometry (MS) was later employed to quantitate celecoxib, but none of its metabolites [33-36]. Zhang et al. used mass spectrometer to characterize the metabolites of celecoxib in rabbit plasma, but did not establish quantitation methods for these metabolites [37]. Here, we propose to establish and validate a comprehensive and sensitive LC-MS/MS method to simultaneously quantitate celecoxib and also its major metabolites, which could be employed in various biological matrices. When this method was applied to the pharmacokinetics study of celecoxib in rat blood, the blood concentration of celecoxib was found to be much higher than previously reported in rat plasma [38]. The divergence between the analyte concentrations in different matrices (blood vs plasma) led us to study the distribution of celecoxib and its metabolites in the plasma and blood cells using this LC-MS/MS method. Additional studies employing this LC-MS/MS method to determine the tissue exposure of celecoxib and its metabolites in humans could aid the understanding of its underlying toxicity [39].

2. Materials and Methods

2.1 Chemicals and reagents

Celecoxib was purchased from LC Laboratory (Woburn, MA). Uridine-5'-diphosphate-glucuronic acid (UDPGA), alamethicin, β -glucuronidase, saccharolactone, magnesium chloride (MgCl_2), potassium permanganate (KMnO_4), hydrochloric acid (HCl), sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), lithium aluminum hydride (LiAlH_4), ammonium acetate (NH_4Ac), potassium dihydrogen phosphate (KH_2PO_4), potassium phosphate dibasic (K_2HPO_4) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, ethyl acetate and water (MS grade) were purchased from EMD (Gibbstown, NJ, USA). Ora-Plus® suspension vehicle was obtained from Professional Compounding Centers of America (Houston, TX). All other materials were used as received. Rat liver microsomes were prepared from Sprague-Dawley rats as previously described [40].

2.1.1 Chemical synthesis and purification of the Phase I metabolites of celecoxib

—Two phase I metabolites of celecoxib, M2 and M3, were generated by chemical synthesis. A mixture of 120mg celecoxib (0.315mmol) and 150mg KMnO_4 in 6mL 0.2M NaOH was refluxed for 2 hours and then cooled to room temperature. 30% NaHSO_3 was added to quench KMnO_4 and the solution was acidified by HCl. The organics was extracted by 10mL ethyl acetate and the extraction was repeated for three times. The combined extract was washed successively with 100mL 0.5N HCl, 100mL water, 100 mL 5% NaHCO_3 and 100mL water, dried over Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure and the residue was purified by Sephadex LH-20 column employing MeOH as the elute solvent. 120.0 mg M3 (0.292mmol) was afforded and the yield was 92.6%. ^1H NMR (500 MHz, $\text{DMSO}-d_6$): δ = 13.3 (br s, 1H), 7.91 (d, J = 8.6 Hz, 2H), 7.85 (d, J = 8.5 Hz, 2H), 7.53 (d, J = 8.8 Hz, 2H), 7.49 (s, 2H), 7.41 (d, J = 8.5 Hz, 2H), 7.32 (s, 1H)

For M2, 100mg M3 (0.243mmol) and 100mg LiAlH₄ were stirred at room temperature in 10mL anhydrous THF for 14 hours. After the remaining LiAlH₄ was quenched by 10mL ice-cold water, the reaction solution was purified as described above to afford 65.3mg M2 (0.164mmol, 67.5% yield). ¹H NMR (500 MHz, DMSO-*d*₆): δ = 7.84 (d, *J* = 8.7 Hz, 2H), 7.56-7.49 (m, 4H), 7.31 (d, *J* = 8.1 Hz, 2H), 7.24 (d, *J* = 8.2 Hz, 2H), 7.18 (s, 1H), 5.28 (t, *J* = 5.9 Hz, 1H), 4.48 (d, *J* = 5.5 Hz, 2H)

1-(4-sulfamoylphenyl)-5-(*p*-tolyl)-1*H*-pyrazole-3-carboxylic acid (Figure 1, B) was prepared as described previously by Uddin et al. [41]. It was employed as the internal standard (I.S.) for the quantitation of celecoxib and its metabolites. ¹H NMR (500 MHz, DMSO-*d*₆): δ = 13.0 (br s, 1H), 7.83 (d, *J* = 8.6 Hz, 2H), 7.50–7.46 (m, 4H), 7.17 (d, *J* = 8.1 Hz, 2H), 7.14 (d, *J* = 8.2 Hz, 2H), 7.01 (s, 1H), 2.27 (s, 3H)

2.1.2 Biosynthesis and purification of the phase II metabolites of celecoxib—

The Phase II metabolites of celecoxib, the glucuronides M1 and M5, were biosynthesized using rat liver microsomes as the resource of uridine 5'-diphospho-glucuronosyltransferases (Ugts) using incubation procedures published earlier [42]. Briefly, rat liver microsomes (final concentration 1mg protein/mL), MgCl₂ (0.88mM), saccharolactone (4.4mM), alamethicin (0.022 mg/mL), UDPGA (3.5mM) and M2 or M3 (100uM) were mixed in a 50mM potassium phosphate buffer (pH 7.4) and incubated at 37° for 4 hours. The incubation volume was 5 mL. At the end of incubation, 1.25mL 94% acetonitrile/6% formic acid was added to stop the reaction. Denatured microsomal protein was removed from the solution by 12000×g centrifugation for 15 minutes. Glucuronides were then purified as described below.

The conjugates were separated from the aglycones and the other gradients in the incubation solution by an Alliance 2690 HPLC equipped with a Waters 2487 dual λ absorbance detector. The conditions were: column, Phenomenex Aqua C18, 5μm, 150mm × 4.6mm (Phenomenex, CA, USA); mobile phase A, 0.1% formic acid; mobile phase B, 100% acetonitrile; gradient, 0-5min, 10% B, 5-15min, 10-40% B, 15-23min, 40-90% B, 23min-28min, 90% B, 28-30min, 90-10% B, 30-35min, 10% B; flow rate, 1mL/min; column temperature, 25°; detect wavelength, 254nm; injection volume, 100μL. The retention times of M1 and M5 are 17.4 and 17.6min, respectively.

The fraction eluted between 17min and 18min was collected and then combined with the same fraction derived from multiple injections. The organic solvent was evaporated under reduced pressure before the solution was frozen and lyophilized. Then the yield was reconstituted in 50μL acetonitrile and used as the standard for M1 or M5. In this chromatography, M1 and its acyl migration products had the same retention time and were eluted simultaneously as a sharp peak from the column. They were collected as a mixture and not further separated because the acyl migration products were also observed in in vivo samples in a previous study [24].

2.2 Instruments and conditions

2.2.1 UPLC conditions—Celecoxib and its metabolites were analyzed on a Waters Acuity™ Ultra-pressure liquid chromatography (UPLC) equipped with a diode array

detector (DAD). The conditions were: column, Waters BEH C18, 1.7 μ m, 100mm $\times$ 2.1mm (Waters, Milford, MA, USA); mobile phase A, 2.5mM NH₄Ac in water, pH 4.5; mobile phase B, 100% acetonitrile; gradient, 0-0.5min, 20% B, 0.5-5.5min, 20-29% B, 5.5-6min, 29-70% B, 6min-6.5min, 95% B, 6.5-7.0min, 95% B, 7.0-7.5min, 95-20% B, 7.5-8min, 20% B; flow rate, 0.5mL/min; column temperature, 45 $^{\circ}$; detect wavelength, 254nm; injection volume, 10 μ L.

2.2.2 UPLC-MS/MS analysis—UPLC-MS/MS analysis was conducted with an API 5500-Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) equipped with a TurboIonSprayTM source at the negative ion mode. The concentrations of celecoxib and its metabolites were determined by multiple reaction monitor (MRM) scan type. The compound-dependent parameters were listed in Table 1, and the main instrument-dependent parameters were set as follows: ionspray voltage, -4500 V; ion source temperature, 700 $^{\circ}$; nebulizer gas, 30 psi; turbo gas, 30 psi; curtain gas, 20 psi.

2.3 Determination of the concentrations of the phase II metabolites

For M1 and M5 prepared from in vitro incubation and HPLC fraction, the concentrations were determined by using the conversion factors of molar extinction coefficients (ECs) of the glucuronides to the corresponding aglycones M2 and M3, respectively. This approach was extensively used to quantify the phase II metabolites of drugs and phytochemicals [43,44].

2.3.1 Identification of M1 and M5 by β -glucuronidase and mass spectrometry—M1 and M5 were diluted in 200 μ L 50mM potassium phosphate buffer (pH=7.4) to a final concentration of 10 μ M, and incubated with β -glucuronidase (1U/mL) at 37 $^{\circ}$ for 10 minutes.

The incubation was terminated by addition of 50 μ L 94% acetonitrile/6% formic acid. The samples were centrifuged and the supernatant was analyzed by UPLC-DAD. M1 and M5 were also identified by ion trap enhanced mass spectra (EMS) scan and product ion (MS2) scan on mass spectrometry.

2.3.2 Determination of conversion factors—Determination of the conversion factors (Ks) was described previously by using UPLC-DAD[44]. Briefly, K is determined by comparing the peak area change of M2 or M3 with that of M1 or M5, respectively, after the glucuronides were hydrolyzed by β -glucuronidase. With K values and the concentrations of aglycones, the concentrations of M1 and M5 were calculated by using the following equations (1) and (2):

$$C_{M1} = C_{M2} / K_{M1} \quad (1)$$

$$C_{M5} = C_{M3} / K_{M5} \quad (2)$$

where C_{M1} , C_{M2} , C_{M3} and C_{M5} were the concentration of M1, M2, M3, M5, respectively and K_{M1} and K_{M5} were the conversion factors for M1 and M5, respectively.

2.4 Pharmacokinetic studies in vivo

2.4.1 Animals and experimental design—The procedures for animal experimentation were approved by University of Houston Institutional Animal Use and Care Committee (IACUC). Male Sprague-Dawley (SD) rats (approximately 250g) were purchased from Harlan Laboratory (Indianapolis, IN) and had been kept in the animal facilities for at least one week before any experiment. The rats (n=6) were fasted for approximately 14 hours with free access to water before the experiment. Celecoxib dispersed in Ora-Plus® suspension vehicle was administered to rats by oral gavage at the dose of 20mg/kg. At 0, 15, 30, 60, 120, 240, 480, 1440, and 2880 min, 60µL of blood was collected in heparinized tubes from rats by tail tip snipping (1-2 mm cut) after the rat was anesthetized by isoflurane. The blood samples were frozen immediately after collection and stored at -80° until analysis.

2.4.2 Standard and quality control samples—Stock solutions of celecoxib, M2 and M3 were prepared in acetonitrile. Stock solutions of M1 and M5 were prepared as described above. To make calibration curves for each analyte, blank rat blood was spiked with stock solutions to reach various concentrations of analytes as follows: 0.3, 1.2, 4.8, 19.5, 78.1, 312.5, 1250, 5000, 20000nM for celecoxib, M2 and M3; 1.5, 5.9, 23.4, 93.8, 375, 1500, 6000nM for M5; 0.5, 2.0, 7.8, 31.3, 125,500, 2000nM for M1. The quality control (QC) samples for each analyte were also prepared at different concentrations: 1, 10, 100, 1000 and 10000 nM for celecoxib, M2 and M3; 5, 50, 500 and 5000 nM for M5; 10, 100 and 1000 nM for M1. All the calibration and quality control samples were stored at -80° until analysis.

2.4.3 Blood sample preparation—40µL blood sample was initially mixed with 40µL saturated NaCl solution (pH 1.5, adjusted by HCl). Then 300µL acetonitrile containing 0.1µM I.S. was added and the mixture was vigorously vortexed for 1 min. The addition of the inorganic salt to blood sample made it immiscible with acetonitrile and the extraction was referred to as “salting-out liquid-liquid extraction” [45]. After 12000×g centrifugation for 15 minutes, the supernatant was transferred to a new tube. The extraction was repeated once and the extract was combined and evaporated to dryness under nitrogen flow. Before analysis the residue was reconstituted in 100µL 25% acetonitrile containing 0.1% formic acid and centrifuged at 12000×g for 15 minutes. 10µL supernatant was injected into the column for quantitative analysis.

2.4.4 Method validation

2.4.4.1 Calibration curve and LLOQ: The standard samples for establishing the calibration curve were prepared and processed as described in the section 2.4.2 and 2.4.3. The peak area ratios of celecoxib and its metabolites to I.S. were calculated and correlated with the concentrations by a least-squares linear regression method with $1/x^2$ weighting. A signal-to-noise ratio of at least 10:1 was used as the principle criterion to determine the lower limit of quantification (LLOQ).

2.4.4.2 Precision and accuracy: In the method validation, the precision and accuracy was examined by quantitating the QC samples for each analyte (at the concentrations described in the section 2.4.2, 6 parallel samples for each concentration). The “inter-day” and “intra-

day” precision and accuracy were calculated from the results of QC sample quantification on the same day and on three different days, respectively.

2.4.4.3 Recovery and matrix effect: QC samples were prepared and processed as described in the section 2.4.2 and 2.4.3, and the same amounts of analytes were added to the extracts of blank rat blood. The peak areas obtained from the two sets of samples were compared correspondingly to determine the extraction recoveries.

For the matrix effects, the same amounts of analytes were added to the extracts of blank rat blood or pure acetonitrile, and then processed as described in the section 2.4.3. The peak areas of analytes were compared correspondingly to determine the matrix effects on the quantification of each analyte.

2.4.4.4 Stability: Stability of celecoxib and its metabolites in rat blood samples were determined by analyzing triplicate QC samples under different storage conditions (at 25° for 2 hours and at –80° for 14 days) or through three freeze-thaw cycles (–80° to 25°).

2.4.5 Data analysis—The pharmacokinetic analysis of celecoxib in rat blood was performed with WinNonlin 5.2 (Pharsight, Mountain View, CA). The non-compartmental approach was used to calculate the pharmacokinetic parameters of celecoxib in rats.

2.5 Comparison of the drug concentrations in whole blood and in plasma

In this study, celecoxib and SD rats (approximately 250g, n=3) were prepared the same way as described in 2.4.1. Before oral administration of celecoxib at 20mg/kg, approximately 150µL blood was collected from each rat and put into heparinized tubes. Then 100µL from the total volume was transferred to another tube and spun at 3000×g for 5min in a centrifuge prewarmed to 39°. After centrifugation, the plasma and blood cells were separated to different tubes immediately and labeled as blank plasma and blood cells, respectively. 4 and 8 hours after celecoxib administration, respectively, another 150µL blood was collected from each rat and processed the same as those collected before oral gavage. Based on a hematocrit of approximately 50% for male SD rats [46], two new sets of “whole blood” were formed as follows: for each rat, 20µL plasma from 4 or 8 hour was mixed with 20µL of blank blood cells, while 20µL of blood cells from 4 or 8 hour was mixed with 20µL blank plasma. All the original and re-mixed blood samples were stored at –80° until analyzed.

3. Results and Discussion

3.1 Chromatography and mass spectrometry

3.1.1 Optimization of UPLC and MS conditions—UPLC and mass spectrometry conditions were optimized to ensure decent peak resolution and shape, and the best sensitivity. For mobile phase A, 2.5mM ammonium acetate buffers (pH from 2.5 to 7.4) or 0.1-0.5% formic acid were tested; for mobile phase B, 100% methanol or 100% acetonitrile were tested. C₈ and C₁₈ columns (Acquity, Waters, 2.1 mm× 100 mm, 1.7µM) were the two candidates for the solid phases. We found that the peaks of M2 and M1 could not be separated from each other when employing a mobile phase A with pH>5, while a mobile phase A with pH<3 usually resulted in bad peak shapes and very long retention time (too

long in fact). Finally, the optimized mobile phase A and B were determined to be 2.5mM ammonium acetate (pH=4.5) and 100% acetonitrile, respectively, which appeared to have achieved the best sensitivity and peak shape. The C₁₈ column was found to be better than C₈ column, with temperature set at 45° to achieve a better separation of M2, M1 and the acyl migration products of M1.

When the MS conditions were optimized, the negative scan mode was selected because the intensity of analyte signals was higher than that in the positive scan mode. Multiple reaction monitoring (MRM) scan type was employed to guarantee the specificity for each analyte. All the compound-dependent and the instrument-dependent parameters were determined by tuning each analyte separately. The peaks of the analytes could be found in a representative chromatogram of blank rat blood samples spiked with celecoxib and its metabolites, with the retention times of 3.18, 4.85, 5.08, 6.67, 7.21 and 7.45 min for the I.S., M5, M1, M2, M3 and celecoxib, respectively (Figure 2, A).

3.1.2 Identification of M1 and M5—The bioconjugates of M2 and M3 prepared from in vitro glucuronidation incubation were treated with β -glucuronidase and analyzed by UPLC-DAD. For the conjugates of M2, the peak with the largest peak area (retention time=4.34min) was identified as M1, which was the 1-*O*-acyl- β glucuronide of M2 (retention time=5.77min), due to its susceptibility to β -glucuronidase (Figure 3, A and B). The other small peaks (retention times=4.09, 4.60 and 4.90min) with the UV spectra (Figure 3, C) similar to M1 were also observed, but they were resistant to the hydrolysis by β -glucuronidase (Figure 3, A and B). They were the acyl migration products (2-, 3- or 4-*O*-acyl- α/β isomers) of M1, which were not β -glucuronidase-sensitive and have been observed for the acyl glucuronides of carboxylic acids [27]. The maximum UV absorbance of M1 and its acyl migration products shifted to longer wavelength (red shift) when compared to that of M2 (Figure 3, C). The peak area of M1 accounted for approximately 80% of the total peak area of all the glucuronides. We also observed a transformation of M1 to its acyl migration products when M1 was diluted in a 37° solution with physiological pH (≈ 7) (data not shown).

After incubation with β -glucuronidase, the peak of M5 (retention time=4.14min) disappeared and resulted in a new peak for M3 (retention time=6.45min) (Figure 3, D and E). M3 and M5 were found to have the same maximum UV absorption wavelength (Figure 3, F).

3.1.3 EC conversion factors of M1 and M5—Using the method described in 2.3.2, EC conversion factors were calculated to be 0.97 and 1.04 for M1 and M5, respectively.

3.1.4 Mass spectrometry chromatogram of celecoxib and its metabolites

Celecoxib and its metabolites were also confirmed by mass spectrometry with single-stage and MS2 full scan. The [M-H]⁻ ions and daughter ions of each analytes were shown in Figure 2. The [M-H]⁻ ion of celecoxib was m/z 380 (Figure 2, B), which is in accordance with its molecule weight. As the oxidation products of celecoxib, the [M-H]⁻ ion of M3 and M2 were m/z 396 and 410, respectively (Figure 2, C-D). The glucuronides M5 and M1

showed m/z 572 and 586, which was 176 Da higher than those of M3 and M2, respectively, indicating that M2 and M5 were the glucuronides (Figure 2, E-F).

3.2 Method validation

3.2.1 Linearity and sensitivity—The method was validated in blank rat blood spiked with the standards of each analyte. The linear range of standard curve was 0.3nM-20000nM for celecoxib, 1.2nM-20000nM for M2, 0.3nM-20000nM for M3, 2.0nM-2000nM for M1, and 1.5nM-60000nM for M5, respectively. The lower limit of quantification was 0.3nM, 1.2nM, 0.3nM, 2.0nM and 1.5nM for celecoxib, M2, M3, M1, and M5, respectively. The LLOQ of celecoxib in this method is 3-500 folds lower than those of previously published assays employing mass spectrometry [33,34,47]. For the metabolites, this is the first LC-MS/MS method for their accurate quantitation. Earlier, Stormer et al. established a HPLC-UV method for quantitation of M2 and M3 in human plasma, but the reported LLOQ values (approximately 25 nM) [48] were much higher than the current LC-MS/MS method.

3.2.2 Accuracy and precision—For each analyte, six replicate QC samples at two or three concentrations were measured to determine the intra-day and inter-day accuracy and precision. The results shown in Table 2 indicate that for both intra-day and inter-day, the accuracies were always between 85-115% and the precisions (variability lower than 15%) were within the acceptable range.

3.2.3 Recovery, matrix effect and stability—Celecoxib and its metabolites were overall extracted efficiently from rat blood samples by the salting-out liquid-liquid extraction method. For each analyte, triplicate QC samples at two or three concentration levels were used to determine the recovery rates and the results were shown in Table 3. For all the analytes at different concentrations, the recoveries were not less than 70%.

For determining the matrix effects of blood samples, peak areas of each analytes were compared when different concentrations of analyte standards were introduced into blood samples before or after the extraction procedures. The salting-out liquid-liquid extraction successfully excluded substances in the blood samples which may decrease the signal intensity of analytes. The results shown in Table 3 indicated that the peak areas of analytes in blood sample extracts were always within 90-110% of those in the neat solutions.

The QC samples were put into various tests to evaluate the stabilities of celecoxib and its metabolites. In general, for all the analytes, the recoveries were found to be no less than 85% in all the stability tests.

3.3 Pharmacokinetic study of celecoxib in rats

After oral administration of 20mg/kg celecoxib in rats ($n=6$), the concentrations of celecoxib and its metabolites in the blood was determined by the validated LC-MS/MS method. The average blood concentrations of celecoxib, M2 and M3 at each time points were shown in Figure 4. M3 was the most abundant metabolite in the rat blood after oral administration, and the concentration of M2 was much lower than that of M3. The pharmacokinetics parameters of celecoxib, M3 and M2 were determined using the non-compartmental model

and shown in Table 4. The $C_{\max}$ of celecoxib, M3 and M2 were 15.40, 7.55 and 0.18 μM , respectively. The $T_{\max}$ of the three analytes were 8.0, 13.3 and 21.3 hour for celecoxib, M3 and M2, respectively. The $\text{AUC}_{0-\infty}$ s of the three analytes were 343.81, 215.41, and 5.35 h μM , respectively. The peaks of M1 and M5 could be observed in the blood samples collected 8 hours after drug administration from 2-3 rats (among a total of 6 rats). However, the concentrations of M1 and M5 were under the LLOQ in all the samples, indicating negligible blood levels of these two metabolites in the pharmacokinetic studies. Paulson et al. analyzed bile samples to study the excretion of celecoxib and its metabolites in rats, and their results demonstrated the presence of M1 and M5 in the bile after oral administration of celecoxib [24]. The evidence appeared to support the notion of preferred excretion of M1 and M5 into the bile after glucuronidation in the liver.

A previous pharmacokinetic study employing [^{14}C]celecoxib showed that in the plasma of healthy male volunteers receiving oral dose of 300mg celecoxib, the predominant metabolite was M2, while the concentration of M3 tended to be only marginal [23]. Meanwhile, in the same study, another major metabolite in human plasma was found to be M1. The divergent pharmacokinetics profiles in the previous and current studies show a species difference in celecoxib disposition between humans and rats. The species difference in glucuronidation activity has long been observed between human and other species [49-51]. It should also be noticed that the previous studies usually selected plasma as the matrix for drug quantitation, which was different from the current study [23,24,38]. To illustrate the importance of matrix selection in the pharmacokinetics study of celecoxib, we conducted the following experiment to determine the distribution of celecoxib and its metabolites in the whole blood vs plasma.

3.4 Comparison of the drug concentrations in whole blood vs plasma

A previous study examined the radioactivity distribution in whole blood and red blood cells after [^{14}C]celecoxib (2mg/kg) was orally administrated to rats [24]. Three hours after oral administration, the tissue to plasma ratio of radioactivity was found to be 4.5 and 6.5 for the whole blood and blood cells, respectively, indicating a prominent partition of celecoxib and/or its metabolites in the rat blood cells (mainly erythrocytes) [24]. We determined the distribution of celecoxib, M2 and M3 in the plasma and blood cells by comparing their concentrations in the whole blood and two sets of re-mixed blood samples. The results were presented in Figure 5. The concentrations of celecoxib in the blood cells were 4.3 and 12.2 folds higher than those in the plasma at 4 and 8hour, respectively. Similar results were also observed for the distribution of M3: the blood cells to plasma ratios of distribution were 12.9 and 35.1 at 4 and 8hour, respectively. For M2, the ratio was 2.3 and 1.98 at 4 and 8hour, respectively. For the total concentrations of celecoxib and its metabolites, the blood cells to plasma ratios were 5.1 and 14.9 at 4 and 8 hour, respectively, consistent with the radioactivity distribution ratio obtained in the previous study [24]. The results also indicated that it may take time for each analyte to reach the equilibrium of distribution between the plasma and the blood cells, due to the fact that the distribution ratios were different at various time points.

In previous pharmacokinetic studies in either rats or human, plasma was usually chosen as the biological matrix to characterize the disposition and metabolism of celecoxib. Unfortunately, the blood cells as an important compartment for drug distribution were inappropriately neglected [52]. Mamidi et al. found that the peak concentration of celecoxib in the plasma was 2.5µg/mL (approximately 7µM) after a single oral dose of as high as 100mg/kg celecoxib in rats, and the T_{max} in the plasma was found to be 3.2 hours [38]. A much higher concentration of celecoxib could be expected in the whole blood than in the plasma. From 4 to 8 hour after oral administration, celecoxib concentration in the plasma decreased from 1.50µM to 0.91µM, while in the blood cells it still increased from 6.36µM to 11.13µM (Figure 5, A). The T_{max} of celecoxib in the whole blood (8 hour) is much longer than that in the plasma, due to the fact that the blood celecoxib concentration was still increasing after plasma T_{max} has been reached. It is likely that the plasma concentration of celecoxib and M3 would be affected by, their accumulation in the blood cells, which would prolong their circulation time in the plasma. The accumulation-release process could have a significant impact on the pharmacokinetic properties of celecoxib and M3. In patients with arthritis, an appropriate recirculating concentration of celecoxib is necessary to maintain therapeutic effects [22,53]. Meanwhile, the cardiovascular toxicity of COX-2 selective inhibitors may be associated with the exposure of the cardiovascular system to the high “circulating” drug concentrations [54,55]. In future studies, the selection of the whole blood as the matrix may help to provide new insights into mechanisms underlying the pharmacodynamics and toxicity of celecoxib.

4. Conclusion

A comprehensive, sensitive and robust UPLC-MS/MS method had been established and validated for the quantitation of celecoxib and its 4 major metabolites in rat blood samples. We found that after oral administration of celecoxib (20mg/kg), the predominant metabolite in rat blood was M3, while the phase II metabolites M1 and M5 were not quantifiable. We also found high blood cells to plasma distribution ratios for celecoxib and M3, which in turn explain why pharmacokinetic profiles of these two compounds should be described using blood instead of plasma concentrations. In future, this UPLC-MS/MS method should also be useful for the quantitation of celecoxib and its 4 metabolites in different biological matrix or bioequivalent buffers.

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The highlights in our studies are

1. Developed a LC-MS method to simultaneously quantitate celecoxib and its 4 metabolites.
2. Validated the sensitive and robust LC-MS method with respect to precision and accuracy.
3. Showed the preferential distribution of celecoxib and its phase I metabolites in the blood cells.
4. Rationalized the use of the blood concentrations to describe their pharmacokinetic profiles.

Figure 1.

Chemical structures, and metabolic or biotransformation pathways involved. (A) The Phase I and II metabolic pathways of celecoxib in rats and humans. (B) The chemical structure of 1-(4-sulfamoylphenyl)-5-(*p*-tolyl)-1*H*-pyrazole-3-carboxylic acid as the internal standard (I.S.). (C) Possible acyl migration products of M1.

Figure 2.

Mass spectrometry chromatogram and spectra of the I.S., celecoxib and its metabolites. (A) The representative MRM chromatography of a blank rat blood samples spiked with each analyte standard. Also shown are MS/MS spectrum of celecoxib (B), M2 (C), M3 (D), M1 (E), M5 (F), and the I.S. (G).

UPLC chromatograms and UV spectra of the metabolites of celecoxib. M1 and its acyl migration products purified from rat liver microsomes incubation was incubated with fresh (A) or boiled (B) β -glucuronidase. Also shown are UV absorbance spectra of M1, the acyl migration products and M2 as the hydrolysis products (C). M5 purified from rat liver microsomes incubation was incubated with fresh (D) or boiled (E) β -glucuronidase. Also shown are UV absorbance spectra of M5 and M3 as the hydrolysis product (F). The retention time (RT) of each analyte determined by the DAD detector was shorter than that in

mass spectrometry chromatogram, due to a larger dead volume between UPLC and mass spectrometry.

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Figure 4.

The blood concentrations of celecoxib (round), M2 (square) and M3 (triangle) after oral administration of 20mg/kg celecoxib in SD rats (n=6). The blood concentration is determined as the average of six samples at each time point and the bars show the standard deviations of the mean. The blood concentration of M1 and M5 were below LLOQ.

Figure 5.

Distribution of celecoxib (A), M3 (B) and M2 (C) in the plasma vs. in the blood cells. 4 and 8 hours after oral administration of 20mg/kg celecoxib in 3 SD rats, blood samples were collected and further processed to form two sets of re-mixed “whole blood” samples (see Materials and Methods for details). Concentrations of each analyte in the original whole blood samples and the reorganized blood samples were then determined. Each bar is an average of three determinations and the error bars are standard deviation of the means.

Table 1

Compound-dependent parameters in UPLC-MS analysis (Q1 = parent ion, Q3 = product ion, DP = declustering potential, EP = entrance potential, CE = collision energy and CXP = collision cell exit potential)

Analyte	Q1(m/z)	Q3(m/z)	Dwell Times (ms)	DP(V)	EP(V)	CE(V)	CXP(V)
Celecoxib	380	316	100	-265	-10	-32	-21
M1	586	410	100	-65	-10	-30	-25
M2	410	366	100	-130	-10	-26	-21
M3	396	302	100	-170	-10	-34	-15
M5	572	396	100	-115	-10	-44	-21
I.S.	356	312	100	-10	-10	-22	-17

Table 2

Method validation for celecoxib and its metabolites in rat whole blood.

Analyte	Linear Range (nM)	Concentration (nM)	Intra-day		Interday	
			Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Celecoxib	0.3-20000	1	110.6	6.6	111.6	6.6
		10	98.5	3.5	98.2	3.7
		100	107.5	8.8	105.6	9.9
		1000	93.3	5.8	100.8	10.8
		10000	89.3	9.9	103.4	10.5
M2	1.2-20000	1	103.9	4.8	101.4	5.1
		10	97.8	3.1	96.5	4.0
		100	108.7	6.9	109.6	6.6
		1000	93.1	3.8	94.8	4.1
		10000	107.5	6.2	109.5	4.8
M3	0.3-20000	1	100.6	5.0	101.0	6.3
		10	94.8	3.2	96.6	3.8
		100	109.8	9.7	114.7	8.8
		1000	100.4	9.9	97.3	11.4
		10000	111.9	6.5	110.2	6.6
M1	2.0-2000	10	94.1	3.7	91.8	6.9
		100	108.0	4.4	105.2	4.4
		1000	92.6	4.8	92.4	4.6
M5	1.5-6000	5	91.5	3.0	92.1	5.4
		50	108.0	8.8	102.7	7.2
		500	93.7	7.5	94.8	8.1
		5000	87.9	5.3	91.6	7.1

Table 3

Recovery, matrix effect and stability of celecoxib and its metabolites in rat whole blood.

Analyte	Concentration (nM)	Recovery	Matrix Effect	Stability		
				25°C 3hours	−80°C 14days	Freeze-thaw 3 cycles
Celecoxib	100	74.5±5.3	97.0±6.8	95.6	93.2	105.2
	1000	73.2±10.35	93.2±8.6	102.3	94.7	92.3
	10000	74.4±5.4	99.1±4.1	94.3	103.9	96.7
M2	100	110.7±10.4	89.7±10.3	91.5	110.0	103.6
	1000	107.7±8.8	91.8±4.5	103.0	103.2	109.7
	10000	95.4±6.3	98.9±1.3	96.4	93.2	101.3
M3	100	100.4±5.3	100.6±13.4	91.2	96.3	97.6
	1000	99.3±5.5	98.4±4.7	96.3	90.3	104.8
	10000	93.7±8.5	100.4±2.5	106.3	107.9	105.6
M1	100	89.6±9.3	96.9±4.2	92.6	94.2	89.6
	1000	87.2±6.6	105.3±7.2	91.3	93.7	95.3
M5	50	82.6±4.5	96.6±4.6	95.3	97.8	109.3
	500	85.9±1.6	102.3±6.3	102.6	96.3	94.2
	5000	81.9±5.3	99.4±9.3	97.8	106.5	107.4

Pharmacokinetics parameters of Celecoxib and its metabolites in rat whole blood after oral administration (20mg/kg, n=6).

Table 4

Analyte	T _{max} (h)	C _{max} (μM)	AUC _{0-∞} (hμM)	T _{1/2} (h)	K _e (h ⁻¹)	V _{ss} (L/kg)	CL (mL/h/kg)	MRT _{inf} (h)
Celecoxib	8.00±0.00	15.40±4.01	343.81±65.75	5.72±1.29	0.13±0.03	1.30±0.47	156.29±29.42	15.09±1.98
M3	13.33±8.26	7.55±1.39	215.41±49.95	-	-	-	-	-
M2	21.33±6.53	0.18±0.07	5.35±1.48	-	-	-	-	-