Short communication

Development of simple and rapid LC–MS/MS method for determination of celecoxib in human plasma and its application to bioequivalence study

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ABSTRACT

A suitable liquid chromatography tandem mass spectrometry (LC–MS/MS) method to determine celecoxib in human plasma is needed for bioequivalence and pharmacokinetic studies of celecoxib preparations. The present study describes a simple, rapid, reproducible, and reliable LC–MS/MS method to determine celecoxib concentrations in human plasma. After one-step liquid–liquid extraction (LLE) using methyl *tert*-butyl ether (MTBE), celecoxib and atorvastatin (internal standard, IS) were eluted on a Luna HILIC column with an isocratic mobile phase, consisting of 10 mM ammonium formate buffer (adjusted to pH 3.0 with formic acid):methanol (5:95, v/v) at a flow rate of 0.2 mL/min. The achieved lower limit of quantitation (LLOQ) was 10 ng/mL (S/N > 10) and the standard calibration curve for celecoxib was linear (correlation coefficients were >0.9995) over the studied concentration range (10–2000 ng/mL). The inter- and intra-assay coefficients of variation ranged from 1.15% to 4.93% and 1.08% to 7.81%, respectively. The chromatographic run time for each plasma sample was <2 min. The developed method was successfully applied to a bioequivalence study of celecoxib in healthy Korean male volunteers.

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1. Introduction

Celecoxib is a non-steroidal anti-inflammatory drug and a selective cyclooxygenase (COX)-2 inhibitor used to treat osteoarthritis, rheumatoid arthritis, acute pain, painful menstruation, and menstrual symptoms with an improved side-effect profile. Celecoxib also reduces the number of colon and rectum polyps in patients with familial adenomatous polyposis [1,2]. Celecoxib is well absorbed and extensively metabolized in humans with <3% of the dose excreted unchanged [3]. Celecoxib is methyl-hydroxylated and further oxidized to the corresponding carboxylic acid, and only a small amount is excreted in the urine as a glucuronide [3]. CYP2C9 is the major enzyme mediating methyl hydroxylation [4,5] and the manufacturer's drug information recommends caution when administering celecoxib to poor CYP2C9 substrate metabolizers, because they might develop abnormally high plasma levels. Although the pharmacokinetic properties of celecoxib in healthy volunteers have been described previously [6–13], no report has been issued for Koreans.

Several analytical methods have been used to determine celecoxib concentrations in human plasma with using various analytical techniques, such as high performance liquid chromatography–ultraviolet detection (HPLC–UV) [6–10,14,15], liquid chromatography–mass spectrometry (LC–MS) [11], and LC tandem mass spectrometry (MS/MS) [12,13]. However, these methods suffer from a number of disadvantages, a large volume of plasma (≥0.5 mL) [6–8,10–12,14], lengthy run times (≥4 min) [6–15] and the lack of an internal standard (IS) [15]. Thus, our objective was to develop a reliable, faster, and high throughput method with simple LLE extraction preparation and a 2 min run time, for the routine determination of celecoxib in human plasma.

The analytical method described in this study was fully validated and successfully used to assess the bioequivalence of two marketed pharmaceutical formulations of 200 mg celecoxib capsules in Koreans.

2. Experimental

2.1. Chemicals and reagents

Celecoxib (100.6% purity) was supplied from Il Hwa Pharm. (Kyunggi-Do, Korea) and atorvastatin calcium was supplied from Sigma–Aldrich (St. Louis, MO, USA), respectively. Methyl *tert*-butyl ether (MTBE) and methanol were obtained from J.T. Baker

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(Phillipsburg, NJ, USA); ammonium formate from Sigma–Aldrich (St. Louis, MO, USA). Distilled water was obtained from an Aqua MAX^{TM}-Ultra water purification system (Young Lin Instrument, Kyuggi-Do, Korea). All other chemicals and solvents were of the highest analytical grades available. The test medication, Celect Capsule [200 mg celecoxib, Il Hwa Pharm. (Kyunggi-Do, Korea)] and the reference medication, Celebrex Capsule [200 mg celecoxib, Pfizer Pharm. (Seoul, Korea)] were supplied in the form of capsules.

2.2. Instrumentation and analytical conditions

The HPLC system was Agilent 1100 series (Agilent, USA). Chromatographic separation was achieved using a Luna HILIC (50 mm × 2.0 mm i.d., 3 μm, Phenomenex, USA) analytical column protected by HILIC guard column (4 mm × 2.0 mm i.d., 3 μm, Phenomenex, USA) at 45 °C. The isocratic mobile phase consisted of methanol–10 mM ammonium formate buffer adjusted to pH 3 with formic acid (95:5, v/v) at a flow rate of 0.2 mL/min. The solution filtered using 0.22 μm membrane and ultrasonically degassed prior to use.

Mass spectrometric detection was performed on a PE Sciex API 2000 triple quadrupole mass spectrometer equipped with an ESI source (AB Sciex, Toronto, Canada). Data acquisition was performed with Analyst^{TM} 1.5 software (AB Sciex, Toronto, Canada). The mass spectrometer was operated in the negative ion mode. Optimized instrument settings specific celecoxib and IS were as follows: curtain gas was 10 psi, ion source gas 1 was 60 psi, ion source gas 2 was 60 psi, ionspray voltage was −4500 V, turbo heater temperature was 500 °C. The precursor ions of celecoxib and IS were formed using declustering potentials of −47 and −35 V, respectively, and their precursor ions were fragmented at collision energies of −27 and −58 eV by collision-activated dissociation with nitrogen at a pressure setting of 5 (arbitrary units). Both quadrupoles were maintained at unit resolution.

2.3. Preparation of standard and quality control (QC) samples

Stock solutions of celecoxib (1 mg/mL) and atorvastatin (IS) (1 mg/mL calculated as free base) were prepared by dissolving the accurately weighed reference compounds in 100% methanol and DMSO, respectively. The stock solution of celecoxib was further diluted with 100% methanol to give a series of standard solution with concentrations of 100, 500, 1000, 2000, 5000, 10,000 and 20,000 ng/mL. A solution of containing 2000 ng/mL of IS was prepared with 100% methanol. Calibration curves were prepared by spiking pooled blank plasma with working solutions to final celecoxib concentrations of 10, 50, 100, 200, 500, 1000 and 2000 ng/mL. Quality control (QC) samples were prepared in the same way at concentrations of 30, 200 and 1600 ng/mL for celecoxib. All the solutions were stored at −20 °C.

2.4. Sample preparation

200 μL volume of human plasma was added to 20 μL of IS (20 μg/mL). This sample solution was extracted with 1.5 mL of MTBE by vortexing. After centrifuged at 20,800 × g at 4 °C, the upper organic layer was transferred to another tube (Microtube, Axygen® Scientific, California, USA) and evaporated to dryness at 50 °C under a gentle stream of nitrogen. The residue was reconstituted in 80 μL of mobile phase by vortexing for 3 min and centrifuged at 20,800 × g at 4 °C for 10 min. Aliquot of this solution (5 μL) was injected into the LC–MS/MS system.

2.5. Method validation

The method was validated for specificity, LLOQ (10 ng/mL), recovery, accuracy, precision, stability and linearity for validation of bioanalytical methods [16].

2.5.1. Specificity and LLOQ

The specificity of the method was measured by analysis of six blank plasma samples of different origin for interference at the retention times of the analyte and IS. Specificity was assessed by comparing chromatograms of a blank human plasma sample, a blank human plasma spiked with celecoxib (10 ng/mL) and IS (20 μg/mL), and human plasma samples of a subject 3 h after an oral administration of 200 mg celecoxib.

2.5.2. Linearity

The linearity of the calibration curves, ranging of 10–2000 ng/mL, was validated with five different calibration curves. The calibration curves ($y = ax + b$) were constructed using the weighted regression method ($1/x^2$) of peak area ratios of celecoxib to IS (y) versus actual concentrations (x).

2.5.3. Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy were performed on the same day ($n = 5$, at each concentration) and on five consecutive days at four concentrations (10, 100, 500 and 2000 ng/mL). Precision is expressed as coefficient of variance (C.V.), at each concentration. The accuracy of the assay was defined as a percentage of the measured concentration over the theoretical concentration. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value, except for LLOQ, which was set at 20%. The LLOQ was determined as the concentrations with a signal to noise (S/N) ratio of 10.

2.5.4. Extraction recovery and matrix effects

The relative recovery, absolute matrix effect and process efficiency was determined at three QC concentrations (30, 200 and 1600 ng/mL, $n = 3$) of celecoxib and IS (20 μg/mL, $n = 9$). The relative recovery was measured by comparing the peak areas obtained from plasma samples spiked before extraction with those from plasma samples spiked after extraction. The absolute matrix effect was measured by comparing the peak response of plasma samples spiked before extraction with those of the pure standards containing equivalent amounts of the celecoxib and IS prepared in mobile phase. The process efficiency was measured by comparing the peak response of plasma samples spiked after extraction with those of the pure standards containing equivalent amounts of the celecoxib and IS prepared in mobile phase.

2.5.5. Stability

The stabilities of celecoxib in human plasma were evaluated by analyzing replicates ($n = 3$, at each concentration) of plasma samples at three QC concentrations (30, 200 and 1600 ng/mL): freeze–thaw stability after 3 freeze–thaw cycles at −70 °C; long-term stability at −70 °C for 2 weeks; short-term stability at room temperature, 4 °C and −70 °C for 24 h; auto-sampler stability at 4 °C for 26 h. In addition, the stability of the celecoxib working solutions was evaluated.

2.6. Application to bioequivalence studies

The validated method was used to determine the plasma concentrations of celecoxib in a bioequivalence study. The subjects were hospitalized (Kyung Hee University Medical Center, Seoul, Korea) and the study protocol was approved by the Korean Food and Drug Administration (KFDA) and the Institutional Review Board of
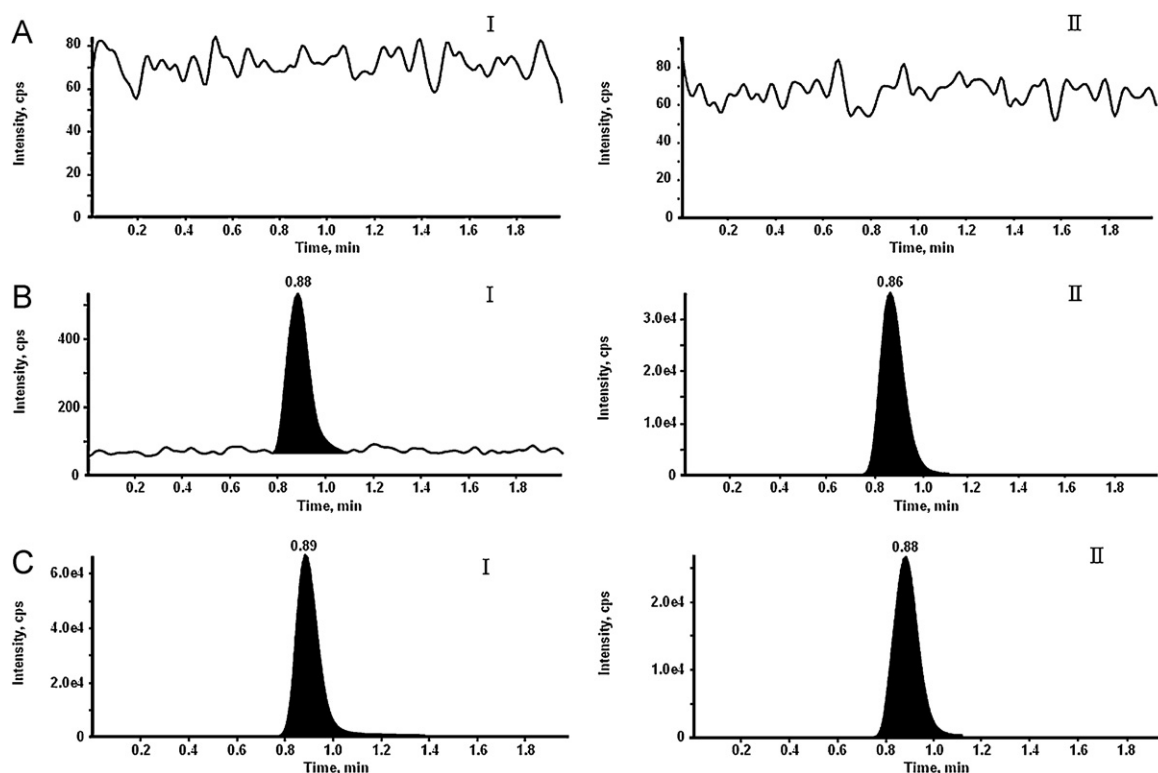
Fig. 1. Chromatogram of (A) blank human plasma, (B) blank human plasma spiked with celecoxib at the LLOQ (10 ng/mL) and IS (20 µg/mL) and (C) plasma sample of a subject 3 h after oral administration of 200 mg celecoxib capsules (measured celecoxib concentration, 1752.551 ng/mL). Peak I, celecoxib; Peak II, IS.

the Kyung Hee University (KMC IRB 1006-03). Thirty-seven healthy Korean male volunteers were given a single oral administration of 200 mg celecoxib capsules with 240 mL of water in a two period randomized crossover study. Their median cubital vein was cannulated and 7 mL blood samples were collected before (0 h) and at 0.5, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.5, 4, 6, 8, 12, 24 and 48 h into sodium heparinized tubes after dosing. Plasma separated by centrifugation at 3000 rpm for 10 min and kept frozen at $-70\,°C$ until analysis.

Pharmacokinetic parameters including extrapolated AUC_∞ (the area under the plasma concentration versus time curve from time 0 to infinity), AUC_{0-48h} (the area under the plasma concentration versus time curve from time 0 to 48 h), C_{max} (peak plasma concentration), T_{max}, elimination half-life ($t_{1/2}$), and elimination rate constant (K_e) were calculated using BA Calc 2007® [17]. For the purpose of bioequivalence analysis AUC_{0-48h} and C_{max} were considered as primary variables. Bioequivalence of two formulations was assessed by means of an analysis of variance (ANOVA) for crossover design and calculating 90% confidence intervals (CIs) of the ratio of test/reference using log-transformed data.

3. Results and discussion

3.1. Method development

To develop a simple and rapid sample preparation, various mobile phases and columns were investigated to reduce the ion suppression induced by endogenous substances and to optimize the shape and retention time of celecoxib and IS. In our extensive preliminary experiments, a C_{18} reverse, CN, cation-exchange and reverse (CR) phase, as well as hydrophilic interaction liquid chromatography (HILIC) columns were tested to optimize celecoxib and IS retention. The best results in terms of separation, peak shape, and reproducibility were obtained with a Luna HILIC

column, whereas CN, CR, and C_{18} reverse-phase columns had peak tailing and splitting with poor sensitivity for celecoxib and IS in >95% organic solutions.

The separation and ionization of celecoxib and IS were affected by the composition of the mobile phase. A series of aqueous mobile phases containing different additives (acidic buffers and additives such as formic acid, ammonium acetate, and ammonium formate) with different pH values were tested with methanol solution. A mobile phase consisting of 10 mM ammonium formate (pH 3.0, adjusted with formic acid) and methanol (5:95, v/v) achieved symmetric peak shapes, and short retention times (Fig. 1). The flow rate of 0.2 mL/min produced good peak shapes and the retention times of celecoxib and IS were 0.88 min and 0.86 min, respectively, with high precision observed for within-batch retention times.

3.2. Method validation

3.2.1. Specificity and LLOQ

Typical chromatograms were obtained using the analytical conditions described above (Fig. 1). No significant interference from the plasma was observed on retention time. The LLOQ (10 ng/mL) was determined with S/N ratios >10.

3.2.2. Linearity

Calibration curves were prepared over the celecoxib concentration range of 10–2000 ng/mL by linear regression using a $1/x^2$ weighting factor. Under our analytical conditions, the calibration curve showed an average linear regression equation ($n = 5$) of $y = 0.00122\ (\pm 0.00006)x + 0.00143\ (\pm 0.00211)$ ($r = 0.9997 \pm 0.00015$), where y is the peak area ratio of the celecoxib to IS and x is the celecoxib concentration. The deviations of all standards from all nominal concentrations were within ±10%.

Table 1
Intra- and inter-day assay precision and accuracy for LC–MS/MS assay of celecoxib in human plasma ($n = 5$).

Theoretical concentration (ng/mL)	Acquired concentration (ng/mL) (mean ± SD)		Precision (% CV)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
10	9.82 ± 0.77	10.14 ± 0.50	7.81	4.93	98.15	101.38
100	101.87 ± 1.97	102.40 ± 1.17	1.94	1.15	101.87	102.40
500	511.56 ± 10.36	510.42 ± 10.21	2.03	2.00	102.31	102.08
2000	2036.80 ± 22.01	2011.08 ± 43.50	1.08	2.16	101.84	100.55

Table 2
Relative recovery, absolute matrix effect and process efficiency of celecoxib using liquid–liquid extraction.

A (% CV)	B (% CV)	C (% CV)	Relative recovery, % RE (C/B) × 100	Absolute matrix effect, % ME (B/A) × 100	Process efficiency, % PE (C/A) × 100
30 ng/mL					
28274.56 (2.3)	22909.01 (2.6)	10461.74 (2.8)	45.67	81.02	37.00
200 ng/mL					
176402.56 (12.7)	145510.44 (2.0)	70392.98 (1.5)	48.38	82.49	39.90
1600 ng/mL					
1306179.65 (3.2)	1053917.43 (0.5)	548595.31 (0.4)	52.05	80.69	42.00

A, mean area response of three replicate samples for celecoxib prepared in mobile phase; *B*, mean area response of three replicate samples for celecoxib prepared by spiking in extracted blank plasma; *C*, mean area response of three replicate samples for celecoxib prepared by spiking before extraction; CV, coefficient of variation.

3.2.3. Precision and accuracy

Intra- and inter-day precision and accuracy of the method are presented in Table 1. The intra-day accuracy ranged from 98.15% to 102.31% and precision ranged from 1.08% to 7.81%. The inter-day accuracy ranged from 100.55% to 102.34% and precision ranged from 1.15% to 4.93%. All results were within the ranges of precision (%) and accuracy (%) specified by the KFDA for bioanalytical applications [16].

3.2.4. Extraction recovery and matrix effects

The purpose of sample preparation is to remove interfering substances and decrease the LLOQ. Accordingly, the procedures must be highly reproducible with high recovery of analyte and a minimum number of working steps. In this study, plasma samples were extracted by one-step LLE using MTBE as the extraction solvent.

The relative recoveries following extraction at three QC concentrations of celecoxib were about 45% (Table 2). The absolute matrix effects and process efficiency were more than 80% and 37%, respectively. In addition, the relative recovery, absolute matrix effect and process efficiency of IS were 65.13 ± 1.71%, 81.12 ± 2.53% and 52.83 ± 3.00% ($n = 9$, mean ± SD) at 20 μg/mL, respectively. These results indicate that no endogenous substances significantly influenced the ion suppression in this analytical method. And this analytical method was reliable to analyze samples for a bioequivalence study.

3.2.5. Stability

The stability of celecoxib in human plasma was investigated by three QC concentrations to cover expected conditions during sample preparation and storage. The analyte was considered stable when stabilities were <±20% of the fresh sample [16]. These results indicate that celecoxib in plasma were stable at room temperature, 4 °C, and −70 °C for 24 h and at −70 °C for 2 weeks (Supplemental Table 1). Celecoxib QC samples in post-extraction matrix were stable under the autosampler conditions (4 °C) for at least 26 h. Celecoxib QC samples were unaffected by three freeze–thaw cycles. The working solutions of celecoxib were found to be stable at room temperature and 4 °C for 24 h and at −20 °C for 2 weeks. No significant deterioration was observed under any of the examined conditions.

3.3. Application to bioequivalence study in healthy volunteers

The devised method was successfully used to analyze approximately 1200 plasma samples in a celecoxib bioequivalence study of
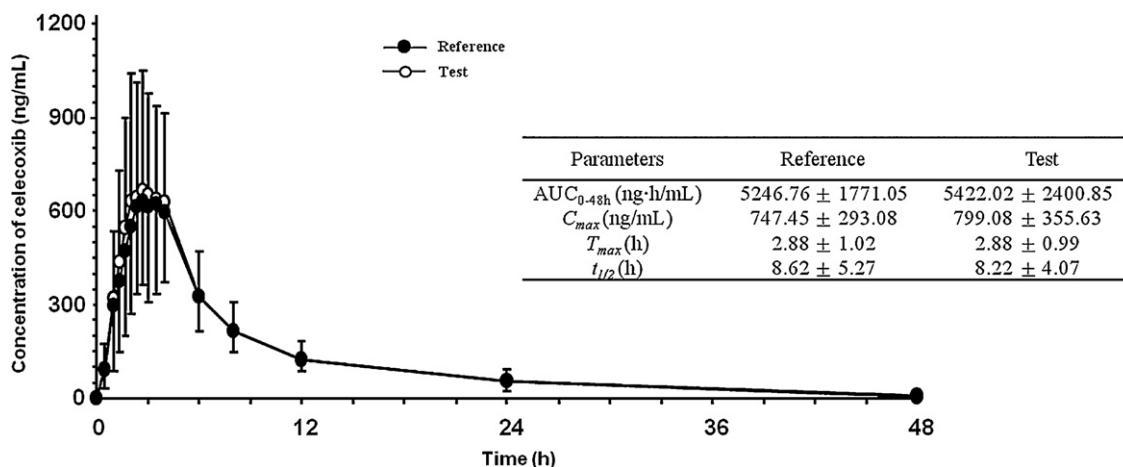
Fig. 2. Mean (±SD) concentration versus time pharmacokinetic profiles of celecoxib from Celebrex capsules (●, reference drug) and Celect capsules (○, test drug) in plasma samples of 37 healthy Korean male volunteers after a single oral administration of 200 mg. Data show the mean and one standard deviation from 37 healthy Korean meal volunteers. Pharmacokinetics parameters obtained were summarized in inset table.

human plasma. The mean plasma concentration–time curve of cele-coxib, after oral administration of 200 mg to healthy Korean male volunteers, is shown in Fig. 2, and the pharmacokinetic parameters derived from these profiles. The 90% confidence intervals for the ratios of area under the curve at 0–48 h (AUC_{0-48h}) and C_{max} were log 0.9444–log 1.1020 and log 0.9368–log 1.1740, respectively, which met the bioequivalence criteria of log 0.80–log 1.25 [16]. The pharmacokinetic values of AUC_t were not similar to a previous study [7], which may have been caused by the influence of CYP2C9 genetic polymorphisms between races [18,19].

4. Conclusions

Here, we described a rapid and convenient method to determine celecoxib in human plasma. The full validation indicated that the established method, with a total running time of 2 min for each sample, was specific, rapid, and reproducible over the concentration range of 10–2000 ng/mL. The devised method met all USFDA guidelines for validating a bioanalytical method [20]. Additionally, this method has been successfully applied to bioequivalence studies of celecoxib to analyze more than 1200 clinical samples. Finally, the devised method reduced analysis time and improved pretreatment compared with previously described methods and was more suitable for the routine measurement of a large number of samples.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.016.

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