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Short communication

Determination of celecoxib in human plasma by high-performance liquid chromatography

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Abstract

A high performance liquid chromatographic method for the quantitation of celecoxib (CEL) in human plasma is presented. The method is based on liquid–liquid extraction with chloroform and reversed-phase chromatography using a Nucleosil CN column (250 mm × 4.6 mm i.d., 5 μm particle size) and UV spectrophotometer detection at 260 nm. The mobile phase consists of acetonitrile:water (60:40 (v/v)). Flutamide was used as internal standard (IS). The assay was linear in the concentration range of 10–1000 ng/ml when 0.5 ml aliquots of plasma were extracted. Within-day and between-day precision expressed by relative standard deviation is less than 4% and inaccuracy does not exceed 3%. The assay was used to analyze samples collected during human clinical studies.

Keywords: Celecoxib; HPLC; Plasma; UV detection

1. Introduction

Celecoxib (CEL), a cyclooxygenase-2 (COX-2) selective inhibitor is a non-steroid anti-inflammatory drug (NSAID) approved for the treatment of rheumatic pain and osteoarthritis [1]. This approval is based on its improved side effects profile in comparison to that observed for NSAIDs, especially during chronic use [2]. Available experimental and clinical data show improved gastric tolerance as compared to conventional, non-selective NSAIDs [3,4]. Selective inhibition of COX-2, the main isoenzyme expressed during inflammation by CEL, is the main specification which reduces the serious side effects of NSAIDs associated with the inhibition of COX-1 seen with non-selective COX inhibitors [5].

A few methods have been presented for quantification of CEL in plasma using high-performance liquid chromatography (HPLC) techniques with UV or fluorescence detection as well as liquid chromatography–mass spectroscopy (LC–MS) [6–10]. Rose et al. [6] introduced a method for the determination of CEL using a normal phase HPLC with UV detection. CEL and its metabolites were separated by a gradi-

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ent reversed-phase HPLC and quantified using UV detection at 254 nm [7]. Pharmacokinetics of CEL was studied in rat by application of a HPLC method assessment [8]. Also a fluorescence detection method for the determination of CEL in human serum by HPLC has been reported [9]. Werner et al. introduced LC–MS method for the analysis of CEL in plasma with selective and high sensitive detection [10]. In this study a simple and sensitive assay for routine analysis of CEL in human plasma has been presented.

2. Experimental

2.1. Chemicals and reagents

Celecoxib (Fig. 1) was extracted from celebrex 200 mg capsules (Searle Ltd., Caguas, Puerto Rico) according to the literature procedure [9]. Internal standard (IS) (flutamide, Fig. 1) was purchased from Merck Sharp & Dohme Research Laboratory (Rahway, NJ, USA). HPLC grade acetonitrile and analytical grade chloroform were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade and used without any further purification.

2.2. Solutions and buffers

Stock solution of CEL was prepared by dissolving 20 mg CEL in methanol to give a final concentration of 2 mg/ml. Standard solutions were obtained by diluting this solution with methanol to give concentrations over the range of 10–1000 ng/ml.

The solution of flutamide, internal standard was prepared by dissolving 20 mg flutamide in methanol to a concentration of 2 mg/ml. The final solution was obtained by diluting this solution with methanol to give concentration of 10 μg/ml of flutamide.

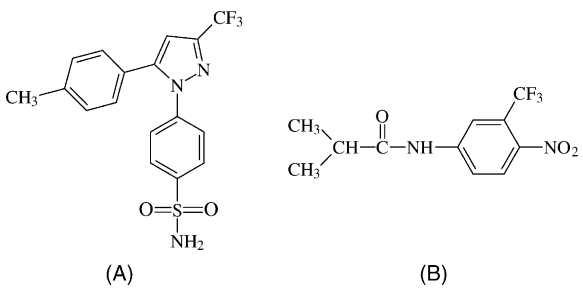

Fig. 1. Chemical structures of (A) CEL and flutamid (B) IS.

Phosphate buffer (pH 5; 0.5 M) was prepared by dissolving 8.7 g potassium phosphate dibasic in distilled water and the pH was adjusted to 5.00 by concentrated orthophosphoric acid, water was added to 100 ml. This solution was used for acidifying and precipitating of plasma proteins. All solutions were stored at 4 °C.

2.3. Chromatographic conditions

The HPLC system consisted of a series 510 pump, a 717 plus Auto sampler, variable 480 UV detector all from Waters (Milford, MA, USA) and multi-channel chromatography data station software Chrom&Spec Version. 1.52 g of sample was used for data acquisition and processing. Separation was achieved using a Nucleosil 100-5 CN analytical column (250 mm × 4.6 mm i.d., Macherey-Nagel, USA). The isocratic mobile phase consisted of water:acetonitrile (60:40 (v/v)) prepared daily and degassed by passing through a 0.45 μm filter. All chromatographic separations were performed at room temperature. The flow rate was set to 0.9 ml/min. The UV detection was performed at 260 nm.

2.4. Sample preparation

Frozen plasma samples were obtained from Blood Transfusion Center (Red Crescent Organization), thawed and allowed to reach room temperature. A 500 μl aliquot of plasma was placed into a test tube, 25 μl of IS solution and 50 μl standard solution of CEL, 50 μl of phosphate buffer (pH 5; 0.5 M), and 4 ml of chloroform were added, respectively. The tubes were vortex mixed for 1 min at 5000 rpm, centrifuged for 10 min at 3500 rpm. Upper layer was discarded and the chloroform layer was transferred to a clean test tube and evaporated to dryness at 50 °C in water bath under a stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase, mixed well and 60 μl of the final clear solution was injected into the HPLC system.

2.5. Quantification

Calibration standards of CEL were prepared by spiking 50 μl of CEL standard solutions and 25 μl of

IS solution to 500 μl of blank human plasma to give final concentrations over the range of 10–1000 ng/ml. The sample extraction and HPLC analysis was performed as described above. Calibration curves were constructed by plotting the measured peak area ratios of CEL to the IS versus concentrations of standard samples. The intra-day (within-run) and inter-day (between-run) accuracy and precision of the method was determined by measuring four replicate samples of CEL standard solutions (10, 100, 400, 1000 ng/ml) on three separate days.

2.6. Extraction yield

Aliquots of 50 μl of CEL standard solutions and 25 μl of IS solution were added separately to two sets of three test tubes. One set was extracted according to the sample preparation method. An aliquot of 100 μl of mobile phase was added to each test tube and 60 μl of the final solution was injected into the HPLC system. The other set was adjusted to 100 μl by mobile phase and 60 μl of each solution was injected for the determination of celecoxib in human plasma by the HPLC system. The peak areas of the two sets were compared. The experiment was repeated on three consecutive days.

3. Results

Typical chromatograms of blank and plasma samples spiked with internal standard and CEL are shown in Fig. 2. Under the chromatographic conditions described, IS and CEL were well resolved in plasma sample and eluted at 6.41 and 7.02 min, respectively following injection into HPLC. Optimization was achieved by monitoring varying reversed-phase columns, mobile systems, flow rate and using a simple one-step extraction method.

The mean recovery of CEL at three different concentrations (10, 400, 1000 ng/ml) and extraction efficacy is showed in Table 1 and determined by comparing peak areas from extracted standard samples with standard solution (10, 400, 1000 ng/ml) in mobile phase was 89%. Flutamide was selected as internal standard due to its acceptable precision and accuracy for CEL determination at the concentration of 250 ng/ml. The recovery of IS was 65%.

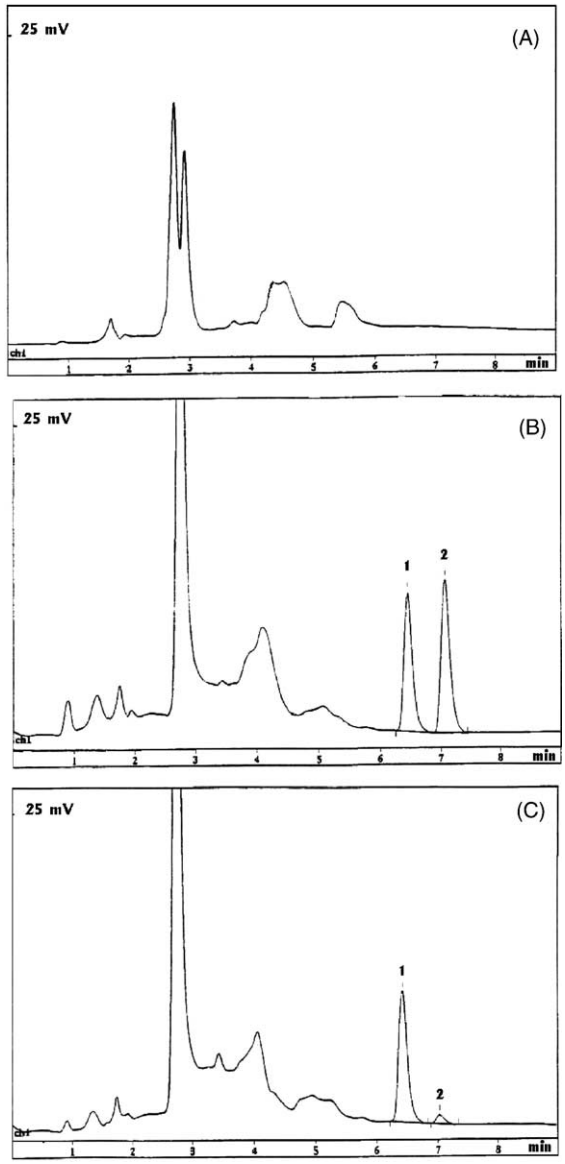

Fig. 2. HPLC chromatograms of CEL and IS (A) Drug free plasma; (B) plasma spiked with CEL (400 ng/ml) and IS (250 ng/ml); and (C) plasma extract from a healthy subject following oral administration of a CEL capsule 100 mg (CEL concentration = 10.12 ng/ml). Peaks: 1, IS; 2, CEL.

3.1. Calibration, accuracy and precision

The standard curve for CEL in different range of concentrations 10, 20, 50, 100, 200, 400, 800 and 1000 ng/ml) in plasma was prepared. The calibration

Table 1
Extraction efficacy of celecoxib from human plasma at various concentrations, mean ± standard deviation ($n = 3$)

Concentration (ng/ml)	Extraction recovery ± S.D. (ng/ml)
10	86.31 ± 3.2
400	88.55 ± 2.4
1000	92.18 ± 1.78

Mean of recovery is 89.01 ± 2.96 ng/ml.

curve displayed excellent linearity ($r^2 > 0.99$) over the concentration range investigated. Typical calibration curve obtained in plasma samples is described by $Y = 0.0033X - 0.0049$, where Y is the peak-area ratio of CEL/IS and X is CEL concentration (ng/ml). Three calibration curves have been examined and the mean value of slopes, intercepts and correlation coefficients are illustrated in Table 2.

The accuracy and precision were determined by preparing five replicate samples of CEL at concentrations of 10, 100, 400, and 1000 ng/ml of plasma on each and three separate days. Concentrations were determined using a calibration curve for each day. Fifteen of blank samples were determined for the method validation. According to the intra-day (within-run) and inter-day (between-run) data good accuracy and precision were observed over the entire concentration range. The results are presented in Table 3. The within-run and between-run variability showed CV values less than 3.56 in all four selected concentrations. The limit of quantification of the method, defined as the minimum concentration that could be measured with a CV <5% was found to be 10 ng/ml in 500 μl of plasma sample. The limit of detection with a S:N ratio of 3:1 was 4 ng/ml in plasma.

3.2. Stability

Stability during repeated freeze-thaw cycles has been demonstrated [6]. Stability of standards and plasma samples were evaluated at −20 °C for 2 months and at a room temperature for 24 h. Under the above conditions, samples preserved their potency (>95%). The present method was used to determine the plasma concentration of celecoxib. Fig. 3 shows the mean ± standard error of the mean (S.E.M.) plasma concentration time profile of CEL. Analysis of CEL concentration of 100 mg celebrex capsule provided the following pharmacokinetic parameters (mean ± S.D.): C_{max} = 940.05 ± 294.23 ng/ml, $AUC_{0-\infty}$ = 5326.19 ± 1282.78 ng h/ml, AUC_{0-t} = 846.80 ± 1454.61 ng h/ml, T_{max} = 2.33 ± 0.72 h, elimination half-life = 7.93 ± 3.25 h (Table 3). The observed values of the pharmacokinetic parameter were comparable to those reported for CEL in previous studies [9].

Table 2
Mean ± standard deviation of slope, intercept, and correlation coefficient of calibration curve equation ($n = 3$)

Slope ± S.D.	0.0033 ± 2.65 ($\times 10^{-4}$)
Intercept±S.D.	0.0049 ± 3.06 ($\times 10^{-4}$)
Correlation coefficient±S.D.	0.9987 ± 6.51 ($\times 10^{-4}$)

Table 3
Accuracy and precision in spiked plasma ($n = 15$; five sets for 3 days)

Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	CV (%)	Error (%)
Intra-day ($n = 5$)			
10	9.84 ± 0.35	3.56	−1.6
100	101.36 ± 0.85	0.84	1.36
400	402.59 ± 1.82	0.45	0.65
1000	1002.11 ± 2.54	0.25	0.21
Inter-day ($n = 15$)			
10	10.25 ± 0.25	2.43	2.5
100	98.77 ± 0.98	0.99	−1.23
400	398.75 ± 2.45	0.61	−0.31
1000	1003.93 ± 3.34	0.33	0.39

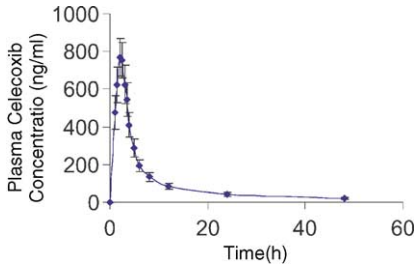

Fig. 3. Mean ± CEL of plasma concentration-time curve following a single oral dose of 100 mg celecoxib to 12 healthy volunteers.

4. Discussion

CEL is a weak acid and could be extracted from an acidic aqueous medium into an organic solvent.

Several extraction methods have been used to accomplish extraction of CEL and its metabolites from biological fluids [7–9]. In the method of Guirguis et al. isooctane-isopropanol as the extraction solvent and C18 column as analytical stationary phase were used. Our experiment showed that a noisy chromatogram was obtained with the above extraction solvent, which decreased the sensitivity of the method. Altering the extraction solvent from isooctane-isopropanol to chloroform, increased the mean recovery of celecoxib from 70 to 89% (Table 2). The extraction procedure described by Schonberger et al. using 0.5 ml plasma, 0.5 ml saturated sodium chloride solution, 1 ml acetonitrile, and 8 ml chloroform with total final volume of 10 ml. This large volume practically caused some difficulties during mixing of samples. In addition, in some cases creation of emulsion prevented the separation of aqueous and organic phase. Furthermore, Schonberger et al. [9] used C18 column that caused peak widening. Chromatograms in Fig. 4 show that switching from C18 to CN column created sharper peak which resulted in an increased sensitivity of the method. A 40% of acetonitrile in water was optimum to achieve the best resolution between IS and CEL peaks. The increased percentage of acetonitrile reduced the retention time of IS and CEL peaks resulting by the interference of the latter and endogenous plasma peaks. Using acetonitrile below 40% gave tailing for IS and CEL peaks. The same behavior was observed when the flow rate adjusted below 0.9 ml/min.

Using UV detection method, we obtained a 10 ng/ml limit of quantification while by using fluorescence detection method 12.5 ng/ml limit of quantification was reported [9].

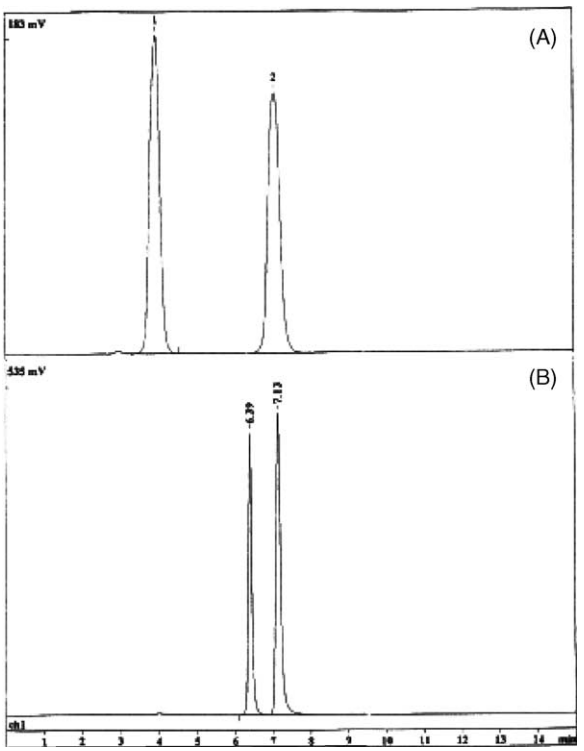

Fig. 4. HPLC chromatogram of CEL and IS on C18 and CN column. (A) The chromatogram of celecoxib (2) and internal standard (1) on C18 column (25 cm, 4 μm) run by water:acetonitrile (40:60 (v/v)) as mobile phase and (B) the chromatogram of internal standard and celecoxib on CN column (25 cm, 4 μm). The mobile phase and scale time are the same.

5. Conclusion

A one-step extraction procedure for CEL from plasma and an improved method for determination of CEL is reported. Compared to previously published methods, the suggested extraction procedure is considerably simple, rapid, reliable, and sensitive. The HPLC technique based on isocratic system and UV detection makes this method suitable to determine small amounts of CEL with good accuracy and reproducibility comparable to the methods performed by gradient reversed-phase liquid chromatography with UV detection [7] and fluorescence detection [9]. Simple sample preparation procedure and a short chromatographic time make this method suitable for processing of multiple samples in a limited amount of time for bioequivalence and pharmacokinetic studies.

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