A rapid and sensitive HPLC method for the analysis of celecoxib in human plasma: application to pharmacokinetic studies

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ABSTRACT

Background and the purpose of the study: A suitable high-performance liquid chromatography (HPLC) method for determination of celecoxib levels in plasma is of prime need for the pharmacokinetics and bioequivalence studies of celecoxib preparations. The present study describes a simple, rapid, sensitive, reliable, and economic HPLC method for determination of celecoxib in human plasma which is more feasible than reported celecoxib HPLC assays.

Methods: The drug and internal standard were extracted using n-hexane /isoamyl alcohol (97:3) and analyzed on a C_{18} µ-Bondapak HPLC column with KH_2PO_4 (0.01M, pH= 4) - acetonitrile (60:40) as the mobile phase, at 260 nm. The method involved simple one-step liquid-liquid extraction procedure with extraction recovery of greater than 90%.

Results: The standard curve covering 0.01-2.0 µg/ml concentration range was linear. The coefficients of variation and relative errors for inter- and intra-day assay ranged from 5.67 to 9.83 and 0.35 to 7.89 %, respectively.

Conclusions: HPLC assay was performed isocratically on a reversed-phase column with UV detection. By this method a limit of quantification of 10 ng/ml of a sample size of 0.5 ml is achieved which is comparable or even better than the reported methods. The developed method was applied to the analysis of celecoxib levels in plasma collected from healthy volunteers who participated in a pharmacokinetic study.

Keywords: Celecoxib, HPLC, Pharmacokinetics, Human Plasma

INTRODUCTION

Celecoxib or 4-[5-(4-methylphenyl)-3- (trifluoro-methyl)-1H-pyrazol-1-yl] benzenesulfonamide is a non-steroidal anti-inflammatory agent which inhibits cyclooxygenase activity (1-3) and is commonly used for pain control and the treatment of rheumatoid arthritis and osteoarthritis (4-7). Celecoxib is well absorbed from the gastro-intestinal tract after oral administration and undergoes extensive metabolism (2).This compound exhibits a terminal half life of 11.2-15.6 h, volume of distribution of 1.7-5.7 l/kg, and protein binding of 97% (1, 2, 8, 9).The effect of food on the absorption of celecoxib is negligible (9). Bioavailability issues have been an increasing concern to drug regulatory authorities once the safety and efficacy of drug products are established (10). A simple and suitable high-performance liquid chromatography (HPLC) method for determination of celecoxib levels in plasma is of prime need for studying the pharmacokinetics of celecoxib. Thus far, a number of HPLC methods have been described to analyze this drug in biological fluids using different clean-up procedures including liquid-liquid extraction (LLE) (11-14), protein precipitation (PP) (15, 16), PP followed by LLE (17, 18), or by solid-phase extraction (SPE) (19), and SPE (20-24). PP method deteriorates the chromatographic column and reduces the sensitivity of the assay. The proposed SPE procedures for celecoxib (20-24) may not be suitable for the processing of multiple samples in a limited amount of time for bioequivalance and pharmacokinetic studies. LLE of this drug from plasma has been associated with low mean recoveries (12), chromatogram with noisy baseline (11), and creation of emulsion (18). HPLC method based on MS or MS-MS (13, 14) for the analysis of the celecoxib in plasma are not readily available in most laboratories.

The present study describes a simple, rapid, sensitive, reliable, and economic HPLC method for determination of celecoxib in human plasma.

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For economical reasons, convenience, and promotion of extraction efficiency, it was appeared of interest to use LLE as clean-up procedure using a mixture of n-hexane and isoamyl alcohol at 97:3 ratios.

MATERIALS AND METHODS

Reagents and Solutions

Celecoxib was supplied by Amin Pharmaceuticals (Isfahan, Iran). Internal standard, flutamide was from Sigma-Aldrich Canada LTD. HPLC grade acetonitrile, methanol and n-hexane were purchased from Caledon (Canada). KH_2PO_4, triethanolamine, isoamyl alcohol, orthophosphoric acid were from Merck (Germany). All reagents and solutions were either HPLC or analytical grades. Water was obtained by double distillation and purified additionally with a Milli-Q system. Brands of oral capsules containing 100 mg of celecoxib and inactive pharmaceutical grade ingredients were supplied by Amin Pharmaceuticals (Isfahan, Iran).

Instrumentation

HPLC method was performed using a Waters 515 HPLC pump with a Rheodyne 7725I autoinjector, Waters 2487 Dual λ absorbance detector and Waters 746 Chromatopac integrator (Waters, USA).

Chromatographic Conditions

A C_{18} µ-Bondapak (250 × 3.9 mm, Waters, Ireland) was used as chromatographic column. The mobile phase consisted of KH_2PO_4 (0.01M) in double distilled deionized water, acetonitrile (40:60), 20 µl of triethnolamine and orthophosphoric acid to obtain final pH of 4 ± 0.1. The aqueous phase was eluted at a flow rate of 1.5 ml/min and effluent was monitored at 260 nm. Quantitation was achieved by measurement of the peak area ratios of the drug to the internal standard. The mobile phase was prepared daily, filtered, degassed by ultrasonication before use and was not allowed to recirculate during the analysis.

Standard solutions

A stock solution of celecoxib was prepared by dissolving 10 mg of celecoxib in 100 ml of methanol in a 100 ml volumetric flask to obtain the concentration of 100 µg/ml. A series of standard solutions at concentrations of 1.0, 2.5, 5.0, 10, 15, and 20 µg/ml were prepared by dilution of the standard solution in methanol to obtain different working solutions and stored at 4°C. Stock solution of flutamide (50 µg/ml) was also prepared in methanol.

Sample Preparation

To 0.5 ml of plasma in a 10 ml test tube, was added 50µl of internal standard solution (50 µg/ml), 50 µl of phosphate buffer (0.5 M, PH = 5) and then the tubes were vortexed for 30 sec. For extraction, 5 ml of the mixture of n-Hexane and isoamyl alcohol (97:3) was added, samples were vortexed for 1 min and centrifuged at 3000 rpm for 15 minutes. The upper layer was then transferred to a clean test tube and evaporated under the nitrogen gas. The residue was reconstituted in 80µl of mobile phase, mixed well and 50 µl of the final clear solution was injected in to the HPLC system.

Calibration curves and quantitation

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at -20°C. Calibration samples of celecoxib were prepared in blank plasma. To separate tubes, were added 0.5 ml of blank plasma, 50 µl of phosphate buffer (PH = 5, 0.5 M), 50µl of internal standard at fixed concentration of 50 µg/ml, 50µl of celecoxib standard solutions at concentrations of 1, 2.5, 5, 10,15, and 20 µg/ml to yield standard celecoxib solutions in the concentrations of 0.01-2 µg/ml. The samples were taken through the preparation procedure described above and an aliquot of 50 µl of the final solution was injected in to the column. Calibration curves were constructed by plotting peak area ratio (y) of celecoxib to the internal standard versus celecoxib concentrations (x). Linear regression was used to quantitate plasma drug concentrations in samples through determination of the peak area of celecoxib to internal standard and comparison of the resulting values with those of the standard curve which was obtained after analysis of calibration samples.

Selectivity and specificity

The presence of possible disturbing endogenous peaks was examined on control human plasma samples obtained from twelve healthy volunteers. These samples were pretreated according to the sample preparation procedure except for the addition of internal standard.

Precision and accuracy

The intra- and inter-day variation of the assay were determined by replicate analysis (n = 5) of calibration samples of celecoxib at concentrations within the range of calibration curve (0.010-2 µg /ml) in a single analytical run on the same day and five different days, respectively, using the same stock solutions and plasma batches. The percent relative standard deviations of results of the assay were determined.

Limit *of Detection (LOD) and Limit of Quantitation (LOQ)*

The parameter LOD was determined using the signal-to-noise ratio by a comparison of the results of the test samples with known concentrations of analyte to blank samples. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. The LOQ was identified as the lowest plasma concentration of the standard curve that could be quantified with acceptable accuracy, precision and variability.

Recovery determination

The recoveries of celecoxib at the concentration range of calibration curve were evaluated by comparison of the peak areas obtained after extraction of known amount of celecoxib from plasma with those obtained from the same amounts of unextracted celecoxib.

Biological samples

The ethics committee on human studies of the Isfahan University of Medical Sciences approved the study. Twelve adult male volunteers who were healthy on the basis of medical history, clinical examinations and laboratory tests, participated in the study. The subjects were instructed to abstain taking any medication at least 2 weeks prior to and during the study period. Informed consent was obtained from the subjects after explaining the nature and purpose of the study. After an overnight fasting, subjects were given a single dose of 200 mg of celecoxib capsules of brand formulation in a randomized fashion with 200 ml of water. Food and drinks (other than water, which was allowed after 2 hrs) were not allowed for 4 hrs after dosing to all volunteers. Approximately 2 ml of blood samples were drawn into heparinized tubes through an indwelling canola before (0 hrs) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 12, 24, 36 and 48 hrs after dosing. The blood samples were centrifuged at 3000 rpm for 15 min; plasma samples were separated and kept frozen at -20^{0} C until the time of the assay.

Pharmacokinetic Analysis

Celecoxib pharmacokinetic parameters were determined by non-compartmental method. The elimination rate constant (k_e) was obtained from the least square fitted terminal log-linear portion of the plasma concentration-time profile. Half-life was calculated by the equation $0.693/K_e$. The area under the curve to the last measurable plasma concentration (AUC_{0-t}) was estimated by the linear trapezoidal rule. The area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by equation of $AUC_{0-t} + C_t / k_e$ where C_t is the last measurable concentration. The peak plasma concentration (C_{max}) and time to peak concentration (T_{max}) were determined by inspection of the individual drug plasma concentration-time profiles.

RESULTS AND DISCUSSION

Fig.1 shows typical chromatograms of blank human plasma (A), blank plasma spiked with flutamide as internal standard and drug at concentration of 1 µg/ml (B), blank plasma spiked with internal standard and drug at concentration of 0.010 µg/ml (C), plasma sample obtained at 2.5 hrs after a single oral dose of 200 mg celecoxib from a healthy volunteer which had celecoxib at concentration of 0.488 µg/ml (D). An optimum flow rate of 1.5 ml/min for the mobile phase resulted in the retention times of 4.8 min for flutamide (internal standard) and 5.8 min for celecoxib. Under the chromatographic conditions described, celecoxib and the internal standard peaks were well resolved and no peak tailing was noticed. No interfering peaks were observed in the chromatogram of blank human plasma or the chromatograms of blank plasma from 12 healthy individuals who participated in the study. Celecoxib is eliminated predominantly by liver and is metabolized in human to three inactive metabolites including hydroxylated, carboxylated, and glucuronic acid conjugate of carboxylated metabolites (24). The authentic metabolite standards were not available to test for any possible interference. However, these metabolites are more hydrophilic than celecoxib, and are unlikely to elute at the same retention time as the parent compound under described chromatographic conditions as reported by Stormer and coworkers (19).

LLE using n-hexane:isoamyl alcohol (97:3) as clean up and extraction procedure, compared to SPE, PP, and some other LLE, resulted in a considerable reduction in peaks corresponding to solvent front, early huge peaks, and interferences from normal plasma constituents.

For the sample preparation in analysis of celecoxib in biological fluids several extraction procedures have been described (11-24). In the two previous reports (15, 16) sample preparation involved PP with a 1:1 ratio of acetonitrile to plasma to avoid dilution of sample and to prevent reduction in the sensitivity of the assay which may leads to inadequate precipitation of the protein contents in the samples and deterioration of the chromatographic column in the long run. SPE procedures for sample clean-up (20-24) may not be suitable for the processing of multiple samples in a limited amount of time for bioequivalance and pharmacokinetic studies. In

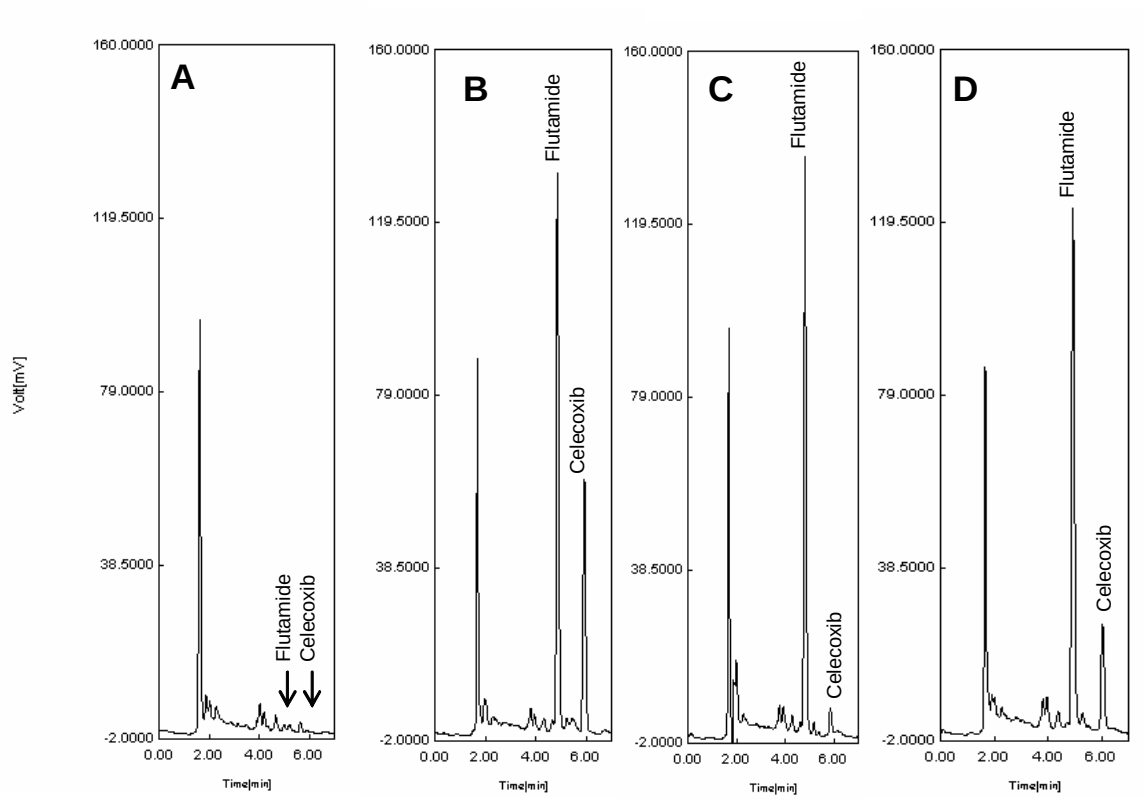

Figure 1. Chromatograms of blank human plasma (A), blank plasma spiked with internal standard, flutamide, and drug at concentration of 1 µg/ml (B), blank plasma spiked with internal standard and drug at concentration of 0.010 µg/ml (C), plasma sample obtained at 2.5 hr after a single oral dose of 200 mg celecoxib from a healthy volunteer containing 0.488 µg/ml of celecoxib (D). The retention times for celecoxib and flutamide are 5.8 and 4.8 min, respectively.

Table 1. Within- and between-days variability of the HPLC assay for determination of celecoxib concentrations in plasma**.**

C (ng/ml)	Within-day variability				Between-day variability			
	Mean	SD	Cv%	Error%	Mean	SD	Cv%	Error%
10	10.43	1.02	9.80	4.28	9.952	0.80	8.03	0.52
25	26.97	2.23	8.25	7.89	26.17	2.11	8.16	4.67
50	49.22	4.59	9.33	1.56	48.37	2.57	5.32	3.26
100	105.2	8.59	8.27	5.17	101.1	8.43	8.34	1.12
250	234.2	13.0	5.67	6.33	231.2	17.9	7.73	7.52
500	491.9	43.1	8.89	1.62	478.4	46.8	9.83	4.31
1000	1034	95.4	9.27	3.46	1033	97.4	9.43	3.31
1500	1501	127	8.56	0.12	1494	102	6.98	0.35
2000	2065	120	5.96	3.27	2034	191	9.43	1.74

SD, Standard Deviation; CV, Coefficient of Variation; Er, Error

the method of Stormer (19) prior PP with acetonitrile, evaporation of the organic phase to dryness and dissolution of the residue in water prior to SPE makes the extraction procedure difficult. Rose et al (22) used more costly normal-phase HPLC in which PP was used prior to SPE. Sensitivity of most HPLC methods using SPE as clean-up procedure have been reported where the limit of quantifications are 25 to 40 ng/ml (22, 24). HPLC methods employing mass spectrometry (13, 14) as the detection system for the analysis of the celecoxib in plasma may be very sensitive and of low quantitation limits, but these methods are not readily available in most laboratories due to their cost and specialty requirements. In the present HPLC method for

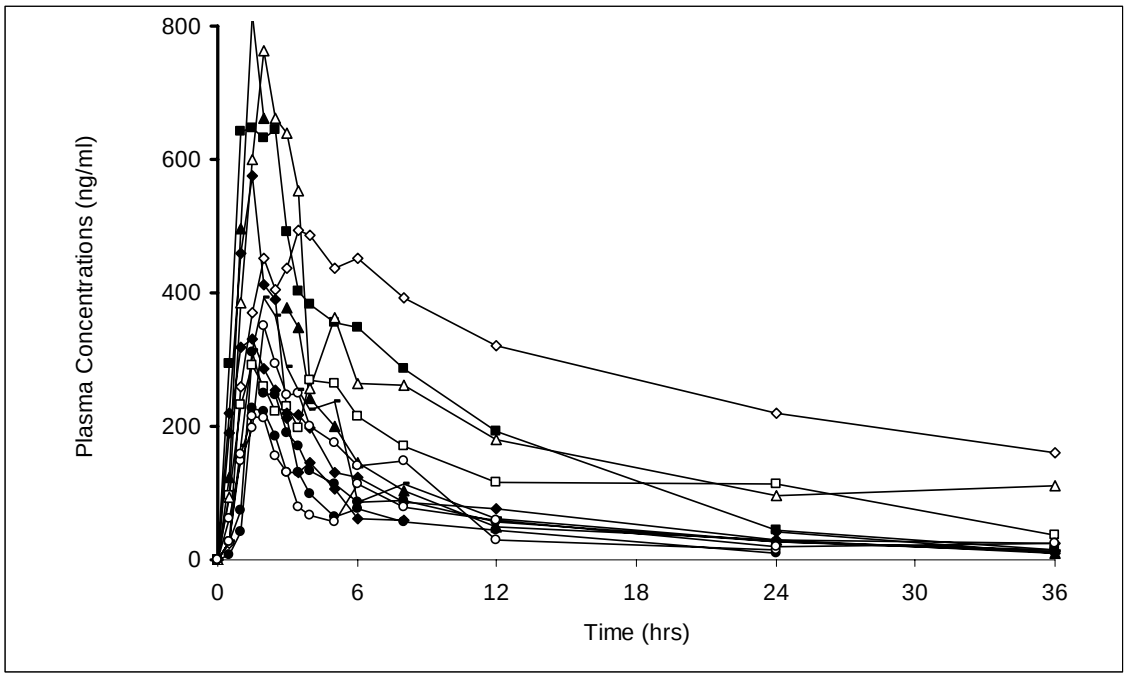

Figure 2. The mean plasma celecoxib levels *vs* time profiles following ingestion of a single dose of 200 mg celecoxib in 12 healthy volunteers.

economic reasons, convenience and promotion of extraction efficiency, a LLE clean-up procedure using a mixture of n-hexane and isoamyl alcohol at the ratio of 97:3 was employed and HPLC was performed isocratically on a reversed-phase column with UV detection. LLE procedure has also some advantageous and disadvantageous. Using isooctane-isopropanol as the extracting solvent (12) has been associated with low mean recovery of about 70%. Application of this extraction procedure has given rise to a chromatogram with noisy baseline resulting in decrease of the sensitivity of the method (11). Using acidic extraction by Schonberger et al (17) as proposed by Gurguis et al (12) resulted in unclear extracts in appearance and the increase in the number of interfering peaks without improvement of the extraction yield. Extraction with chloroform increased mean recovery of celecoxib to about 90% (11), however, using large volume of chloroform (8 ml) for low volume (0.5 ml) of plasma sample practically caused some difficulties in mixing of samples (18). In addition, in some instances creation of emulsion prevented the separation of aqueous and organic phases and reduced extraction efficiency (18). In addition, in some cases, the aqueous phase which can not be completely removed diffuses to the organic solvent and results in interfering peaks in the chromatogram. The extraction procedure of this assay is economical and convenience with good extraction efficiency as was determined by comparison of the peak areas of the spiked plasma samples with those of un-extracted celecoxib solution. The mean recoveries were found to be 91.7% for celecoxib which was significantly higher than previous methods in which LLE was used as extraction and clean-up procedure (11, 12, 18). The recoveries were consistent for the samples as demonstrated by R.S.D. of less than 3.2%. Recoveries were not dependent on the concentration, which consequently resulted in good linearity of the calibration curve. Good Linear relationships were found when the peak area ratios of celecoxib to the internal standard were plotted versus the celecoxib plasma in the concentration range of 0.01 to 2 µg/ml. The linear regression equation for the concentration range were Y = 0.0005 X (± 0.00004) + 0.00106 (± 0.00009). Intra- and inter-day reproducibility for calibration curves were determined on the same day in replicate (n = 5) and on different days for four consecutive days (n = 20) using same pooled plasma sample. The corresponding mean (± S.D.) coefficient of the linear regression analysis was 0.9997 ± 0.0075. The results of within- and between-day variability are presented in table 1. The percent of CV ranged from 5.67 to 9.83 and relative errors were within 0.35 to 7.89 %. Results of coefficients of variation and percent errors indicate that method is reproducible within day and between days. The detection limit for celecoxib was approximately 0.003 µg/ml at a signal to noise ratio of 3:1 and the limit of

Table 2. Pharmacokinetic parameters following administration of 200 mg celecoxib to 12 healthy volunteers.

Prameters	(Mean ± SEM)
C_{max} (ng/ml)	450.50 ± 59.40
AUC_{0-48} (ng.h/ml)	4248.42 ± 1558.07
$AUC_{0-\infty}$ (ng.h/ml)	4501.17 ± 1145.33
T_{max} (hrs)	1.87 ± 0.18
$T_{1/2}$ (hrs)	12.76 ± 1.44

$T_{1/2}$, elimination half-life; AUC_{0-48}, area under the curve to the last measurable concentration; $AUC_{0-\infty}$, area under the curve extrapolated to infinity; C_{max}, peak plasma concentration; T_{max} , time to peak plasma concentration

quantitation corresponding with a coefficient of variation of less than 10% was 0.010 µg/ml using half a ml plasma sample which was comparable or to some extent higher than some of the previously reported methods using UV or fluorescence detectors (13, 18, 20, 24). Celecoxib was measurable at the first sampling time (0.5 h) and after 4 half-lives in all volunteers. The use of internal standard increases the accuracy of the assay whose availability is an important issue in HPLC assays. Stability of celecoxib was determined for spiked plasma samples under the conditions which have been described previously (11). The results indicated that the samples were stable and preserved their potency during processing and sample preparation conditions. Post-preparative stability studies have also shown that celecoxib is stable in processed sample up to 72 hrs at room temperature. Previous studies have shown that celecoxib is stable in serum samples for at least 1 year, during storage at -20°C (12, 19). It has also been reported that it was stable following three cycles of freeze-thaw and long-term storage (11, 22). The mean concentration-time profile following oral administration of 200 mg celecoxib are plotted in Fig. 2. The mean pharmacokinetic parameters are also summarized in Table 2. These pharmacokinetic parameters are in good agreement with those reported previously (11, 13, 15, 18).

CONCLUSION

In summary, an isocratic reversed-phase HPLC method based on single-step LLE with UV detection was used for the determination of celecoxib in human plasma. Compared to previous reports, the proposed extraction procedure is considerably efficient, simple and rapid. The limit of quantification was found to be 10 ng/ml with 0.5 ml plasma sample and the sensitivity of the method is comparable or higher than other similar reported HPLC methods. Good precision, accuracy, simplicity, shorter time of analysis and sufficient sensitivity of the method makes it particularly useful for processing of multiple samples in a limited period of time for pharmacokinetic studies of celecoxib.

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