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Determination of celecoxib in human plasma using solid-phase extraction and high-performance liquid chromatography

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Abstract

A simple reversed phase high-performance liquid chromatography (HPLC) method was developed for determination of celecoxib levels in human plasma. The procedure involves solid-phase extraction of celecoxib and the internal standard (SC-236) from plasma using C_{18} extraction cartridges. The chromatographic separation of celecoxib and SC-236 was achieved with a Nova Pak C_8 column (3.8 mm × 150 mm) eluted with a mobile phase consisting of acetonitrile–tetrahydrofuran–sodium acetate buffer (pH 5.0) in the ratio of 30:8:62. An ultraviolet light detector with the wavelength set at 215 nm was employed for detection. Celecoxib was well resolved from the plasma constituents and the internal standard. The extraction recovery of celecoxib and SC-236 from human plasma was greater than 88%. Linear calibration curves were established over a concentration range of 40–4000 ng/ml when 0.25 ml aliquots of plasma were used. The inter- and intra-day R.S.D. for the assay was less than 12 and 5%, respectively. This assay has been applied to the analysis of celecoxib levels in plasma samples collected from healthy participants entered into a Phase II clinical study.

Keywords: Celecoxib; High-performance liquid chromatography; Human plasma

1. Introduction

Celecoxib (Celebrex®; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide; Fig. 1) is a selective cyclooxygenase-2 (COX-2) inhibitor approved for relief of the signs and symptoms of inflammation associated with rheumatoid arthritis and osteoarthritis [1]. Because it preferentially inhibits COX-2 but not cyclooxygenase-1 (COX-1) at therapeutic doses in humans, celecoxib provides an improved safety profile in comparison with conventional non-steroidal antiinflammatory drugs which inhibit both cyclooxygenases [1]. Because COX-2 is overexpressed in a broad range of pre-malignant, malignant and metastatic human epithelial cancers [2], clinical studies are ongoing to evaluate the use of selective COX-2 inhibitors such as celecoxib in cancer prevention and chemotherapy. A recent study showed that celecoxib can reduce the polyps formation in patients with familial adenomatous polyposis [3] and has led to the approval of celecoxib as adjuvant treatment of familial adenomatous polyposis. In preparation for

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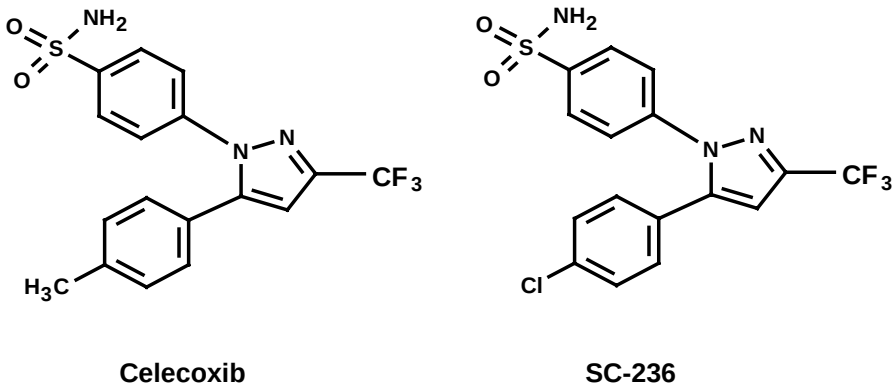

Fig. 1. Chemical structures of celecoxib and SC-236 (internal standard).

sporadic colon adenoma prevention trials planned at our institution, we have developed and validated a reversed phase high-performance liquid chromatography (HPLC) procedure for determination of celecoxib levels in human plasma. This method is intended for monitoring adherence to the study medication and examining the patient-to-patient variations in plasma celecoxib levels. A number of analytical procedures for determination of celecoxib in human plasma have been reported recently. One used a more costly normal phase chromatographic system [4] and the others employed mass spectrometry as the detection system [5–7] which may not be readily available in academic laboratories. Employing a less costly reversed phase system and a commonly available detection system, our method provides analyte resolution, assay precision, and assay accuracy similar to the published procedures [4–7].

2. Experimental

2.1. Chemicals and reagents

Celecoxib and SC-236 were kindly supplied by Pharmacia Corp. (Peapack, NJ, USA). Acetic acid (Baker Analyzed ACS Reagent) was obtained from J.T. Baker (Phillipsburg, NJ, USA). HPLC grade ammonium acetate, sodium acetate trihydrate were purchased from Fisher Scientific (Pittsburgh, PA, USA). HPLC grade acetonitrile and tetrahydrofuran were purchased from EM Science (Gibbstown, NJ, USA). An extraction buffer (0.1 M ammonium acetate) was prepared fresh daily by dissolving 0.7708 g ammonium acetate in 100 ml Milli-Q water. A sodium acetate buffer (0.02 M, pH 5.0) was prepared fresh daily by mixing 0.02 M sodium acetate (2.722 g sodium acetate in 1 l of Milli-Q water) and 0.02 M acetic acid (1.201 ml glacial acetic acid in 1 l of Milli-Q water) in the ratio of 67.8:32.2, respectively. The mobile phase consisted of 0.02 M sodium acetate buffer (pH 5.0), acetonitrile, and tetrahydrofuran in the ratio of 62:30:8. The volume of each mobile phase component was measured separately. All the three components were then combined and filtered (0.2 μm nylon membrane, Alltech Associates, Deerfield, IL, USA) to prepare the mobile phase.

2.2. Working solutions

Celecoxib and SC-236 (internal standard) stock solutions with concentrations of 1 mg/ml were prepared in methanol and stored at −20 °C before use. The celecoxib stock solution was serially diluted in methanol to working solutions in concentrations of 0.1, 0.25, 0.5, 1.25, 2.5, 5, and 10 μg/ml. SC-236 stock solution was diluted in methanol to a working solution of 1 μg/ml. When stored at −20 °C, all stock solutions and working solutions were found to be stable for at least 3 months. For preparation of calibration standards and quality control standards, appropriate aliquots of working solutions were evaporated to dryness and reconstituted in blank human plasma.

2.3. Instrument

HPLC was performed with a chromatographic system (Waters Associates, Milford, MA, USA) consisted of a Model 717 autosampler, a Model 501 pump, a Model 486 variable wavelength UV detector, and an automated chromatographic data acquisition system (Millenium^{32}, Version 2.15.01). The centrifugal evaporator was Savant Model AS 160 (Farmingdale, NY, USA).

2.4. Sample preparation/extraction

To prepare a standard, 100 μl of the celecoxib working solution (100–10,000 ng/ml) and 150 μl SC-236 working solution (1000 ng/ml) were evaporated to dryness and then reconstituted with 250 μl of blank human plasma. To prepare a sample, 150 μl SC-236 working solution (1000 ng/ml) were evaporated to dryness and then reconstituted with 250 μl of plasma sample. Two milliliters of 0.1 M ammonium acetate solution were mixed with the plasma sample or calibration standard before applying onto the solid-phase extraction cartridges. BakerBond Octadecyl SPE cartridges (100 mg, JT Baker, Phillipsburg, NJ, USA) were pre-conditioned with methanol, water, and ammonium acetate (0.1 M) before the samples were applied. Following sample application, the cartridges were washed with 2 ml of 0.1 M ammonium acetate and allowed to be vacuum dried completely. Celecoxib and SC-236 were eluted with 150 μl methanol twice. The eluent was dried in a centrifugal evaporator. The residues were reconstituted with 350 μl mobile phase and centrifuged at 10,000 × *g* for 5 min. Three hundred microliters were injected onto the HPLC.

2.5. Chromatographic conditions

Reversed phase HPLC separation was carried out using a Nova Pak C_8 column (5 μm, 150 mm × 3.9 mm; Waters Associates) at room temperature. Use of the analytical column was preceded by that of a direct-connect column pre-filter (Alltech Associates). The mobile phase consisted of acetonitrile, tetrahydrofuran, and 0.02 M sodium acetate buffer in the ratio of 30:8:62. The flow rate of the mobile phase was 1.5 ml/min. The column eluent was monitored at a wavelength of 215 nm. The run time of each injection was set at 25 min.

2.6. Validation procedures

The calibration curve consisted of a blank sample (blank human plasma), a zero sample (blank human plasma spiked with the internal standard), and blank human plasma spiked with different celecoxib concentrations and a fixed concentration of the internal standard (final plasma celecoxib concentrations of 40, 100, 200, 500, 1000, 2000, and 4000 ng/ml). Calibration standards were prepared on each analysis day from a single batch of celecoxib/internal standard working solutions. The linearity of the method was evaluated with single determination of blank, zero, and each of the seven different celecoxib concentration standards. Calibration curves were constructed using the peak area ratios between celecoxib and the internal standard versus the theoretical celecoxib concentration of the spiked plasma standard. The data were analyzed by unweighted linear least-squares regression and the intercept, slope, and correlation coefficient were determined. The variability of slopes and intercepts of the calibration curves were determined by constructing the curves on five different days.

Blank plasma samples from six different individuals with no specific dietary restrictions were extracted as described above to test for potential interference from biological specimens. The analytical method was also evaluated to assess intra- and inter-day variations at celecoxib concentrations of 40, 1000, and 4000 ng/ml. These quality control standards were prepared on each analysis day from a single batch of celecoxib/internal standard working solutions. Five determinations were performed for each concentration and the analysis was repeated over five different assay days. The concentrations of the quality control standards were determined from the calibration curve prepared on the same day. The R.S.D. of the concentration measured within a run (five replicates) and among five different runs was used to determine the intra- and inter-day precision of the assay, respectively, and was determined as

$$\text{R.S.D.} = \frac{\text{standard deviation}}{\text{mean measured concentration}} \times 100$$

The percent difference between measured and spiked concentrations determined within a run and

among five different runs was used to determine the intra- and inter-day accuracy of the assay, respectively, and was determined as

$$\%\,\text{difference} = \frac{\text{measured} - \text{added concentration}}{\text{added concentration}} \times 100$$

The recovery of celecoxib was determined by comparing the peak area of the extracted quality control standards at 40, 1000, and 4000 ng/ml with unextracted standards that represent 100% recovery. The effects of freeze and thaw on the stability of celecoxib were determined in plasma spiked with celecoxib to final concentrations of 40, 1000, and 4000 ng/ml. The plasma standards were stored at −70 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were transferred back to −70 °C and kept refrozen for 24 h. The freezing and thawing cycle was repeated two more times, and analyzed after the third cycle. The effects of long-term storage on the stability of celecoxib were determined in plasma spiked with celecoxib to final concentrations of 40, 1000, and 4000 ng/ml. The plasma standards were stored in cryotubes in separate aliquots at −70 °C until analysis at 0, 1, 2, 3, and 9 months after storage.

3. Results and discussion

The UV absorption spectrum of celecoxib indicated a peak at 252 nm, however, higher absorbance was observed at below 215 nm. In order to improve the sensitivity of the assay, we performed our analysis at 215 nm. Because of the low wavelength used, a mobile phase composed of acetonitrile and sodium acetate buffer was initially employed to minimize background absorbance. Satisfactory resolution between celecoxib and SC-236 was obtained. However, endogenous components originating from blank human plasma interferred with the celecoxib determination. The addition of a small percentage of tetrahydrofuran further improved resolution between celecoxib and SC-236 and separated the interferring peaks from the analytes. Under our assay condition, larger analyte peak-to-noise ratios were found at 215 nm than at 252 nm. Fig. 2 shows typical chromatograms of blank plasma and plasma spiked with celecoxib and SC-236. No interferring peaks were observed in the chromatogram of blank human plasma shown or in the chromatograms of blank plasma from five other individuals who had followed no specific dietary or medication restrictions. Celecoxib and SC-236 were well resolved and the retention times were around 18 and 21.5 min, respectively. Fig. 3 illustrates plasma samples obtained from two different individuals after taking 400 mg celecoxib once daily for 2 weeks. For both individuals, the blood sample was collected at 12 h after the last celecoxib dose. Celecoxib concentration was determined to be 120 ng/ml in one subject and 1441 ng/ml in the other. Other than the celecoxib peak, no additional peaks were identified in the clinical samples shown and in other clinical samples analyzed. Celecoxib has been reported to be eliminated predominantly by hepatic metabolism [8]. The metabolism of celecoxib was found to involve hydroxylation of celecoxib at the methyl moiety to form a primary alcohol and by further oxidation of the hydroxyl group to form a carboxylic acid metabolite [8]. The carboxylic acid metabolite of celecoxib was subsequently conjugated with glucuronic acid to form the 1-*O*-glucuronide [8]. We did not have the authentic metabolite standards available to test for interference. However, because these metabolites are more hydrophilic than celecoxib and SC-236, they may not retain on the solid-phase extraction cartridges or the analytical column under our assay conditions, or may elute early in the chromatogram as shown previously using radiolabeled chemicals [8].

To further evaluate the specificity of the assay, we have tested 19 frequently used medications for potential interference. These include acetaminophen, allopurinol, amitriptyline, cimetidine, ciprofloxacin, diclofenac, digoxin, estradiol, ibuprofen, metoprolol, naproxen, nifedipine, nortriptyline, phenobarbital, propranolol, theophylline, triamcinolone, simvastatin, and verapamil. None of the compounds tested was found to co-elute with celecoxib and the internal standard.

Plasma calibration curves were constructed with the peak area ratios of celecoxib to the internal standard and celecoxib concentrations. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient (r^2) of the calibration curve. The calibration curve was found to be linear over the range of 40–4000 ng/ml. The linearity was found to be quite satisfactory and reproducible with time. The average slope and intercept of the regression equation

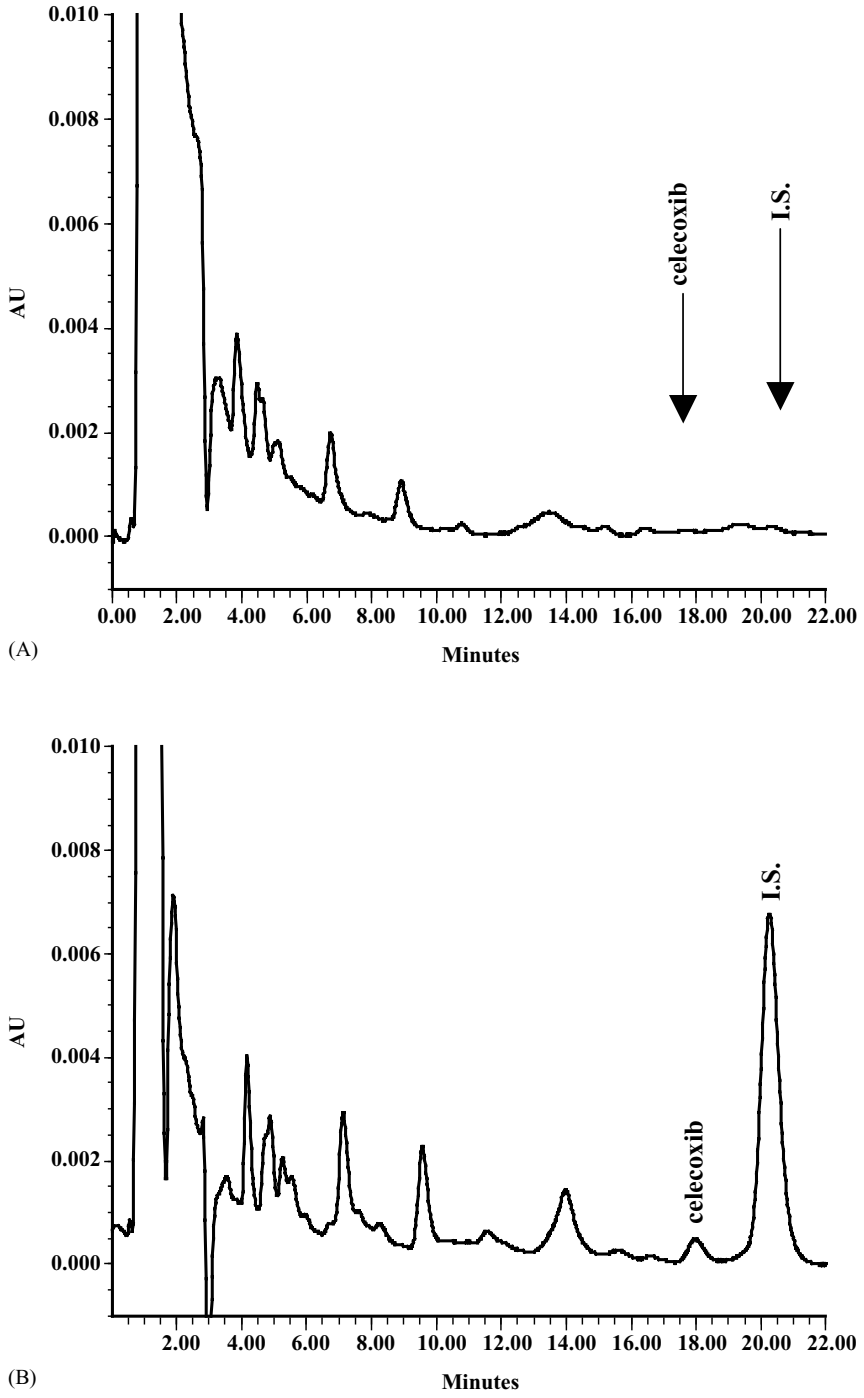

Fig. 2. Representative chromatograms of (A) blank human plasma and (B) a plasma standard containing 40 ng/ml celecoxib and 600 ng/ml internal standard (SC-236).

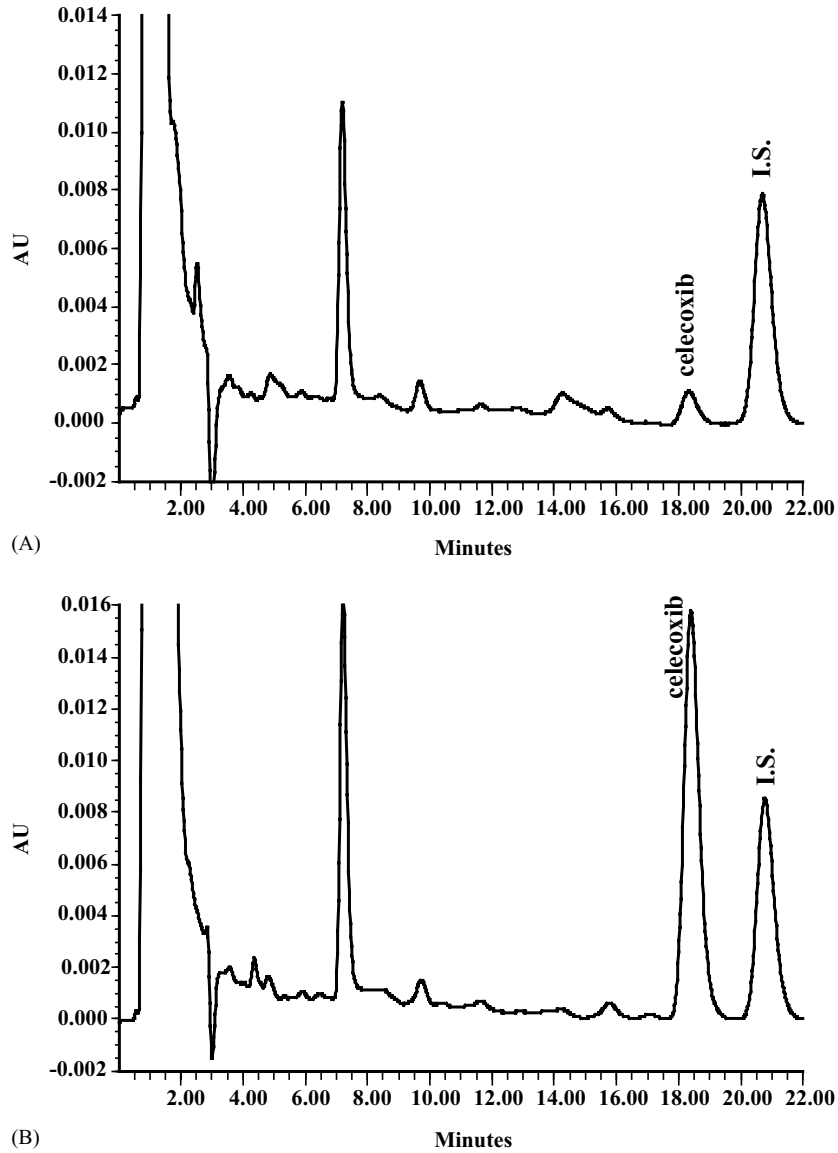

Fig. 3. Chromatograms of plasma samples obtained from two different subjects after receiving 400 mg celecoxib once a day for 2 weeks. For both individuals, the blood sample was collected at 12 h after the last celecoxib dose. The plasma celecoxib concentration was determined to be 120 ng/ml in (A) and 1441 ng/ml in (B).

was 0.0022 ± 0.0002 ml/ng and -0.028 ± 0.02 ($n = 5$), respectively. The r^2 values ranged from 0.996 to 0.999. The limit of quantification (LOQ) was determined as the lowest concentration on the calibration curve that showed a peak response at least five times the response compared to blank response and yielded a R.S.D. of less than 20% and an accuracy of 80–120% of nominal concentration. Based on these criteria, the LOQ was determined to be 40 ng/ml with 0.25 ml of plasma.

Table 1 summarizes the assay precision, accuracy, and extraction efficiency. The inter- and intra-day variation of the assay was determined at celecoxib concentrations of 40, 1000, and 4000 ng/ml. Five replicates

Table 1
Intra- and inter-day variation of the analysis

Concentration added (ng/ml)	Inter-day variation ($n = 5$)			Intra-day variation ($n = 5$)			Recovery ($n = 3$)
	Mean concentration found (ng/ml)	Accuracy[a] (% difference)	Precision[b] (% R.S.D.)	Mean concentration found (ng/ml)	Accuracy (% difference)	Precision (% R.S.D.)	(% recovered[c])
40	45	11.7	11.4	44	11.4	1.9	96
1000	1006	0.6	1.1	997	−0.3	2.2	89
4000	4088	2.2	3.1	3996	−0.1	4.9	89
Internal standard[d]	–	–	–	–	–	–	91

[a] Expressed as % difference: [(concentration found − concentration added)/concentration added] × 100.
[b] Expressed as R.S.D.: (S.D./mean) × 100.
[c] Expressed as mean peak area of extracted samples/mean peak area of the unextracted samples.
[d] At a plasma concentration of 600 ng/ml.

of each concentration were analyzed on each analysis day and the analysis was repeated over five different assay days. The inter-day precision of the assay over five different assay days, as measured by % R.S.D., was less than 12% for the concentrations tested. The inter-day accuracy of the assay, as measured by % difference, was less than 12%. The intra-day assay variation was determined for each of the assay day and results from the day with the largest variation were reported, with % R.S.D. and % difference less than 5 and 12%, respectively. Extraction recoveries for celecoxib were greater than 88% at all tested concentrations. The extraction recovery for the internal standard was 91%.

The stability of celecoxib in plasma following freeze–thaw and long-term storage was determined at three concentrations (40, 1000, and 4000 ng/ml). Consistent with that reported previously [4], celecoxib

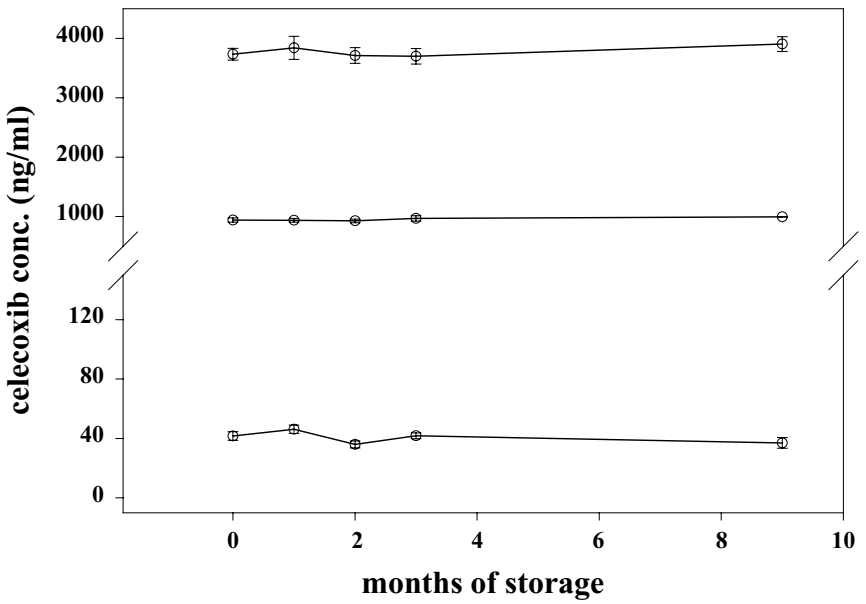

Fig. 4. Effects of long-term storage on the stability of celecoxib in human plasma. Plasma samples at celecoxib concentrations of 40, 1000, and 4000 ng/ml were prepared and stored in cryotubes at −70 °C. Triplicate analysis of each concentration was performed following 0, 1, 2, 3, and 9 months of storage.

was stable following three cycles of freeze–thaw. The long-term storage effect on the stability of celecoxib in human plasma is shown in Fig. 4. There were no significant changes in celecoxib concentrations when the plasma samples have been stored in cryotubes at −70 °C for up to 9 months. A recent study has shown that celecoxib is stable in serum samples for at least 1 year, during storage at −20 °C [9]. For post-preparative stability, celecoxib and the internal standard have been found to be stable in processed samples over 72 h in the autosampler (maintained at room temperature). We have not specifically determined the stability of the analyte during sample collection and sample handling. In reported celecoxib pharmacokinetic studies [9,10], blood samples were centrifuged within 30 min of collection and plasma separated and stored at −20 or −80 °C until analysis. For the ruggedness, our method has been found to be reproducible with a significant time lapse between periods of operation and from one analyst to another. The chromatographic resolution and peak response were stable after injection of at least 500 extracted plasma standards/samples.

The assay has been applied to clinical samples collected from a Phase II trial. Sixty-eight healthy subjects received 400 mg dose of celecoxib once daily for 2 weeks. The study was designed to collect a fasting blood sample at 12 h following the last celecoxib dose. Detailed study description and results will be reported separately. The average plasma celecoxib level was found to be 607 ± 338 ng/ml, with values ranging from 82 to 1700 ng/ml. The large variation observed in the celecoxib levels is partly attributed to deviations in the actual sample collection time, with the elapsed time between the last celecoxib dose and the time of blood collection ranging from 9 to 24 h. In addition, large inherent inter-individual differences in celecoxib metabolism have been noted [11] and may contribute to the observed variations.

4. Conclusion

A reversed phase HPLC assay has been developed for determination of celecoxib in human plasma. The LOQ was 40 ng/ml with 0.25 ml of plasma sample. The method has been found to be precise, accurate, and suitable for the analysis of plasma samples collected in clinical intervention trials.

Acknowledgements

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