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Determination of celecoxib in human plasma by normal-phase high-performance liquid chromatography with column switching and ultraviolet absorbance detection

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Abstract

A method is described for the determination of celecoxib in human plasma. Samples were extracted using 3M Empore membrane extraction cartridges and separated under normal-phase HPLC conditions using a Nucleosil-NO_2 (150×4.6 mm, 5 μm) column. Detection was accomplished using UV absorbance at 260 nm. The HPLC method included a column switching procedure, in which late eluting compounds were diverted to waste, to reduce run-time to 12 min. The assay was linear in the concentration range of 25–2000 ng/ml when 1-ml aliquots of plasma were extracted. Recoveries of celecoxib were greater than 91% over the calibration curve range. Intraday precision and accuracy for this assay were 5.7% C.V. or better and within 2.3% of nominal, respectively. The assay was used to analyze samples collected during human clinical studies. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Cyclooxygenase 2 (COX-2) inhibitors have the potential to displace nonsteroidal antiinflammatory drugs (NSAIDs) as the major treatment choice for the relief of inflammation and pain [1]. This potential is based on their improved side effect profile in comparison to that observed for NSAIDs, especially during chronic use [2]. Such side effects include gastrointestinal lesions and the occurrence of gastrointestinal ulceration and bleeding; these effects are suspected to be in the large part caused by the coinhibition of cyclooxygenase 1 (COX-1). Whereas COX-2 is associated with inflammation, pain and pyretic responses, COX-1 is an enzyme found in healthy tissues and plays a role in thrombosis and in the homeostasis of the gastrointestinal tract and kidneys.

Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide, Fig. 1) is a COX-2 inhibitor that has recently received marketing approval in several countries [3–5]. A method for the determination of celecoxib in human plasma was required in order to support human pharmacokinetic/pharmacodynamic studies. No methods for the analysis of celecoxib in human plasma have appeared, to date, in the literature. An existing method [6] for the determination of rofecoxib, another COX-2 inhibitor, in human plasma could

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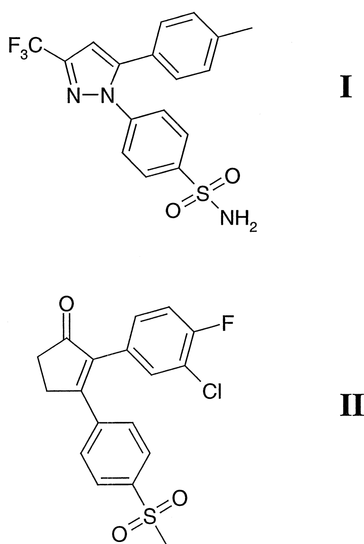

Fig. 1. Structure of celecoxib (**I**), the internal standard (**II**).

not be applied for the analysis of celecoxib due to the marked difference in physical and chemical properties between the two compounds; specifically the postcolumn photochemical derivatization/fluorescence detection scheme that was used in the rofecoxib assay was not applicable to celecoxib. A normal-phase HPLC method using column switching with a nitrophenyl column and UV detection for the determination of celecoxib in human plasma is the subject of this publication.

Although reversed-phase chromatography dominates in HPLC column applications [7], numerous normal-phase chromatography columns are available for separations that may not be easily obtained under reversed-phase conditions. Normal-phase packings containing a nitrophenyl functional group exhibit unique selectivities with respect to their ability to separate compounds possessing double bonds, in particular, aromatic substituents. Selectivity on these columns is influenced by the charge transfer interaction between the electron-withdrawing nitrophenyl groups on the stationary phase and the electron-rich aromatic groups of the compounds being separated [8,9]. Nitrophenyl columns are typically utilized to separate polycyclic aromatic hydrocarbons [10]. Few other uses that take advantage of the unique characteristics of this stationary phase appear in the literature [11–15]. The example presented here illustrates the application of a nitrophenyl column operated in the normal-phase mode for the determination of celecoxib in plasma samples originating from human clinical studies.

2. Experimental

2.1. Materials

Celecoxib was obtained from the Chemical Data Department of Merck Research Labs. (Rahway, NJ, USA). The internal standard (I.S., Fig. 1) was provided by the Medicinal Chemistry Department of Merck Frosst Canada (Kirkland, Quebec, Canada). Solvents (Omnisolve, HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Drug-free human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA). All other reagents were of ACS grade and were used as received. Empore™ high-performance extraction disk cartridges (C_{18}-SD, 10 mm/6 ml) were obtained from 3M (St. Paul, MN, USA).

2.2. Instrumentation

The HPLC system consisted of two Perkin-Elmer (Norwalk, CT, USA) Series 200 LC quartenary pumps operated in isocratic mode, a Perkin-Elmer Series 200 autosampler with an optional Peltier Accessory tray set at 19°C and an Applied Biosystems (Foster City, CA, USA) Model 785A Programmable Absorbance detector. Column switching was conducted using a Valco (Houston, TX, USA) 10-port switching valve. Two positions on the switching valve were jumpered to functionally create a six-port valve. The analog output of the detector was connected to a PE-Nelson (Cupertino, CA, USA) ACCESS*CHROM Data System via a PE-Nelson 900 Series interface. The switching valve was controlled via contact closures on the interface that were triggered at the appropriate times by the ACCESS*CHROM data acquisition method. Absorption and fluorescence spectra were obtained using a diode-array spectrometer (HP 8452, Hewlett-Packard, Palo Alto, CA, USA) and an Hitachi (Danbury, CT, USA) Model F-4500 spectrofluorometer.

2.3. Chromatographic conditions

The mobile phase in both pumps was the same and consisted of hexane–methylene chloride–isopropyl alcohol (70:25:5, v/v). Upon mixing, the mobile phase was used without additional treatment (e.g., filtering, degassing). The flow-rate for both HPLC pumps was 1.4 ml/min. Column 1 was a Nucleosil-NO_2 (10×3.2 mm, 5 μm) cartridge column and Column 2 was a Nucleosil-NO_2 (150×4.6 mm, 5 μm) analytical column, both manufactured by ES Industries (West Berlin, NJ, USA). Column 1 was replaced after approximately 75 injections to maintain a high peak efficiency. Column 2 had a lifetime of over 1000 injections. The analytical column temperature was maintained at 27°C using a column heater. The sample injection volume was 65 μl. UV detection was performed at 260 nm.

2.4. Switching valve programming

A column switching procedure [16] was used to eliminate interferences from late eluting endogenous peaks and thus reduce run time. Valve switching position 1 was set upon injection causing both column 1 (10×3.2 mm) and column 2 (150×4.6 mm) to be in line with the detector. After 0.8 min, switching position 2 was used to divert late eluting peaks remaining on column 1 to waste. Flow through column 2 was maintained in line with the detector. The two pumps maintained a flow-rate of 1.4 ml/min through both columns during the total run time of 12 min. The valve was reset to position 1 immediately upon injection of the next sample. No modification of the column switching time was required over the duration of the application of the assay.

2.5. Preparation of standards

A 250 μg/ml stock solution of celecoxib was prepared by weighing 2.5 mg of reference material into a 10-ml volumetric flask and diluting to volume with acetonitrile–water (50:50). This stock solution was subsequently used in the preparation of working standards of 40, 30, 20, 10, 4, 2, 1, and 0.5 μg/ml by dilution using acetonitrile–water (50:50). A stock of the internal standard was prepared by the addition of 1 mg of reference material into a 10-ml volumetric flask and diluting to volume with acetonitrile–water (50:50). This stock was diluted with acetonitrile–water (50:50) to prepare a 10 μg/ml solution. Working standards of celecoxib and internal standard in acetonitrile–water (50:50) were stable for at least 2 months when stored at room temperature. Standards of celecoxib in plasma were prepared by adding 50 μl of each working standard to 1 ml of drug-free human plasma. The resulting standards were used to quantitate clinical plasma samples containing celecoxib over the concentration range of 25–2000 ng/ml.

2.6. Plasma extraction procedure

A 1-ml aliquot of plasma was pipetted into a Sarstedt (Newton, NC, USA) 15-ml polypropylene conical tube. A 25-μl aliquot of 10 μg/ml working internal standard solution was pipetted into each of the tubes containing the samples and the previously prepared standards. Tubes containing samples received an additional aliquot of acetonitrile (50 μl) to make these samples equivalent in organic content to the standards. The tubes were vortexed. Four 250-μl aliquots of acetonitrile were added to each tube with vortexing for 10 s after each addition. The tubes were capped and centrifuged (2500 *g*) for 10 min. The supernatant was decanted into a disposable 16×100-mm glass culture tube containing 3 ml of 0.10 *M*, pH 3.0, sodium phosphate buffer and mixed. The buffered samples were poured into individual 10 mm/6 ml 3M Empore C_{18} solid-phase extraction cartridges positioned on a 20-place vacuum manifold equipped with stopcocks at each position. SPE columns for plasma were conditioned using sequential washes of 2 ml acetonitrile and 2 ml water. Stopcocks were used during conditioning to halt flow of solvents just before the liquid level reached the top of the prefilter. Upon transfer of the sample to the conditioned cartridge, the stopcocks remained open until the cartridges were removed from the vacuum manifold. The buffered plasma was aspirated through the SPE column using a vacuum pressure of 2700–3300 Pa. The cartridges were rinsed with 1 ml of water and 1 ml of acetonitrile–water (25:75). The cartridges were allowed to aspirate to dryness after which they were removed from

the manifold and suspended into 15-ml disposable centrifuge tubes. The analytes were eluted from the SPE cartridge by drawing two 1-ml aliquots of acetonitrile through each cartridge using centrifugation (150 *g*, 3 min). Elution using centrifugation as opposed to vacuum was observed to provide a more consistent recovery of the analyte from the Empore SPE cartridges. The tube containing the elution solvent was placed in a Zymark (Hopkinton, MA, USA) Turbovap LV evaporator, and the solvent was evaporated using nitrogen (50°C, 15 p.s.i., 30 min). The residue in the tube was reconstituted using 250 μl of mobile phase and transferred to a low-volume conical glass vial prior to injection onto the HPLC.

3. Results and discussion

3.1. Spectroscopic properties of celecoxib

The UV-Vis absorption spectrum of celecoxib indicated the presence of an analytically useful absorption band with a maximum at 252 nm (ε= 18 500 M^{-1} cm^{-1}).

The spectrum was unaffected by changes in pH over the range of 3–12. Celecoxib was found to exhibit weak fluorescence with a maximum of emission at 358 nm when excited at 252 nm. Fluorescence spectra were practically the same at both pH 3 and 7 (acetonitrile–buffer, 50:50), but emission intensity was reduced in the same solvent at pH 12. In contrast to rofecoxib [6], the UV and fluorescence spectra for celecoxib were observed to be unaffected by exposure to UV light for up to 2 min when irradiated in 1-cm quartz cells in a 'merry-go-round' photochemical reactor equipped with four 254-nm lamps.

Based on the spectral characterization results, an assay utilizing UV absorbance detection was developed. Although the λ_{max} of celecoxib was 252 nm, utilization of a detection wavelength of 260 nm was found to reduce UV detector baseline drift attributed to the combination of HPLC pump flow fluctuations and residual UV absorbance of the normal-phase chromatographic mobile phase. This change was observed to have minimal effect on the limit of quantitation for celecoxib.

3.2. Chromatographic system development

Initially, satisfactory peak shape and retention factor (k) were obtained for celecoxib under reversed-phase HPLC conditions using an Inertsil ODS-2 column with a mobile phase consisting of acetonitrile–phosphate buffer (65:35, v/v%, pH 7.0). However, these HPLC conditions failed to separate celecoxib from peaks originating from endogenous compounds in two of five lots of control plasma when the samples were prepared using solid-phase extraction (SPE).

As an alternative to reversed-phase chromatography, Nucleosil-NO_2 and Chromegabond Nitro HPLC columns were evaluated under normal-phase conditions for their ability to resolve the analyte from endogenous components in the plasma extracts. Celecoxib, under normal-phase conditions, would be anticipated to interact with the nitro columns through a charge transfer mechanism between the aromatic groups on the analyte and the nitrophenyl groups on the column packing. It was hoped that the unique selectivity provided by the charge transfer interaction would enable celecoxib to be resolved from endogenous components in the plasma extracts.

Both columns were observed to provide adequate peak shape and retention of the analyte and internal standard. The Chromegabond Nitro column, with a mobile phase hexane–methylene chloride–isopropyl alcohol (65:25:10), was found, however, to be ineffective in resolving the analytes from endogenous peaks in human control plasma extracts prepared via SPE.

Chromatograms of control plasma extracts obtained using the Nucleosil-NO_2 column with a mobile phase of hexane–methylene chloride–isopropyl alcohol (70:25:5) were free of interferences at the retention times of celecoxib and internal standard; hence, this column was chosen for the assay. Under these conditions, celecoxib eluted with k=3.4, while the internal standard eluted at k=2.3.

It is interesting to note that the analytes were overly retained (k>20) on the Chromegabond column under the mobile phase conditions utilized for the Nucleosil column. Additionally, the elution order of celecoxib and internal standard was reversed on the Chromegabond column as compared with the Nucleosil column. Thus, although both columns

contained nitrophenyl-bonded silica, the retention characteristics of the two columns varied significantly, presumably due to differences in secondary interactions between the two base silicas and the analytes.

Although chromatograms of plasma extracts on the Nucleosil column were free of interferences at the retention times of celecoxib and internal standard, late eluting peaks originating from the plasma matrix were observed which necessitated an extended chromatographic run time of 20 min. Previously in such cases we have utilized a column switching system in order to decrease run time [16]. The utilization of such a system with the celecoxib assay allowed the analysis time to be reduced to 12 min, which resulted in a significant improvement in assay throughput.

3.3. Extraction procedure development

Initial experiments to develop a suitable sample preparation procedure focused on the use of solid-phase extraction, as this technique is much more readily automatable compared with liquid–liquid extraction. Celecoxib was found to extract with good efficiency from plasma buffered to pH 3 using an Empore C_{18} extraction cartridge. The pK_a of pyrazole is reported to be 2.52 [17], hence the pyrazole group of celecoxib would be expected to be predominately un-ionized at pH 3, thus leading to the high observed extraction recovery at this pH. The recovery of the internal standard, however, under these conditions was highly variable. The inconsistent recovery of internal standard was attributed to the high protein binding of this compound. Addition of a protein precipitation step with acetonitrile prior to extraction significantly improved the consistency of extraction of internal standard, making it more analogous to that observed for celecoxib.

3.4. Extraction recovery

Recovery of the extraction procedure was determined for celecoxib at low (50 ng/ml), middle (500 ng/ml) and high (1500 ng/ml) concentrations on the calibration curve. The recovery of internal standard was determined at its working concentration of 250 ng/ml. Recoveries were calculated by comparing the absolute peak heights of the standards in human plasma prepared as per the assay procedure, to neat standards evaporated and reconstituted in the same fashion as the extracted samples. The results are listed in Table 1. Recoveries for celecoxib were greater than 91% at all tested concentrations. The recovery for the internal standard was 86%.

3.5. Assay selectivity

Fig. 2 shows chromatograms of extracted control plasma and a plasma standard spiked to contain celecoxib and internal standard at concentrations of 25 and 250 ng/ml, respectively. Fig. 3 shows pre- and post-dose chromatograms from a subject receiving celecoxib. The selectivity of the assay is illustrated by the fact that no endogenous peaks are present at the retention times of celecoxib or internal standard in either the control or pre-dose chromatograms.

3.6. Linearity

Weighted (weighting factor$=1/y$, where y=peak height) least-squares regression calibration curves were constructed by plotting the peak height ratios of

Table 1
Intraday precision, accuracy and extraction recovery for the determination of celecoxib in human plasma

Nominal conc. (ng/ml)	Celecoxib determined conc. mean (ng/ml, $n=5$)	Accuracy[a] (%)	Precision[b] (%)	Recovery[c] (%, $n=5$)
25	24.7	98.8	4.8	–
50	49.8	99.6	4.1	93.4
100	100.8	100.8	3.5	–
200	204.5	102.3	5.5	–
500	495.5	99.1	5.0	91.3
1000	1000.2	100.0	4.1	–
1500	1522.1	101.5	5.7	96.1
2000	1987.9	99.4	4.2	–
I.S.[d]	–	–	–	85.7

[a] Expressed as [(mean observed concentration)/(nominal concentration)]×100.

[b] Coefficient of variation of peak height ratio of celecoxib/internal standard.

[c] Expressed as [(mean peak height of extracted samples)/(mean peak height of neat standards)]×100.

[d] At a concentration of 250 ng/ml.

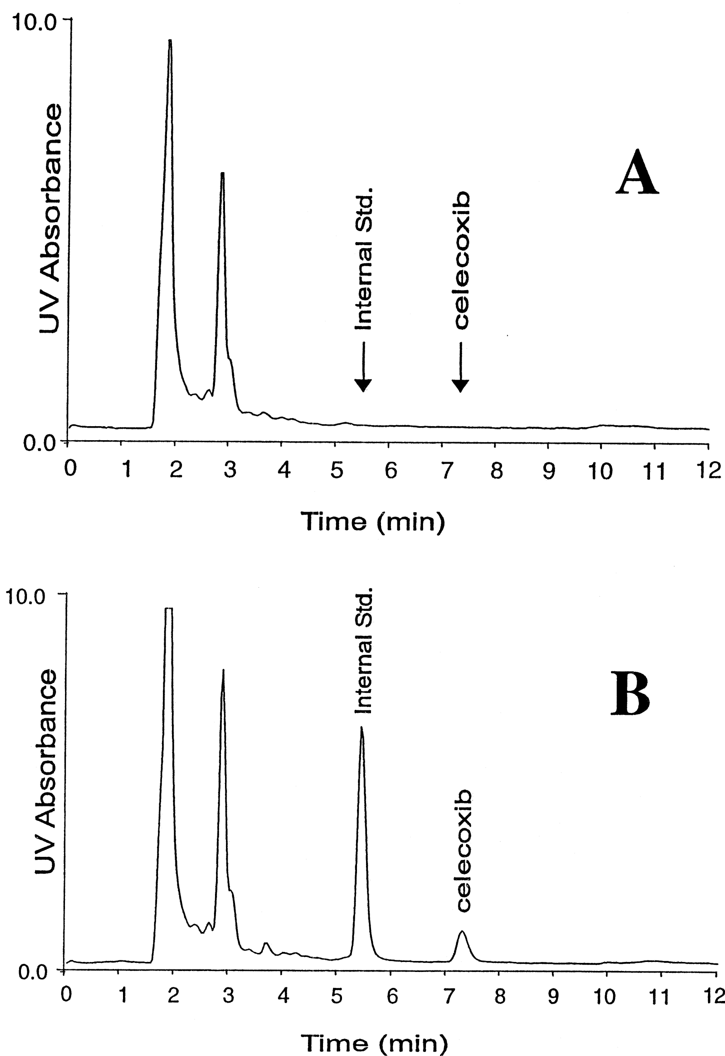

Fig. 2. Representative chromatograms of (A) control human plasma and (B) a plasma standard containing 25 ng/ml celecoxib and 250 ng/ml internal standard.

analyte to internal standard versus standard concentration. The use of the weighted least-squares regression resulted in 2.3% or less deviation between the nominal standard concentrations and the experimentally determined concentrations calculated from the regression equations, for all concentrations on the standard curve.

3.7. Assay precision and accuracy

An assessment of the intra-day variability of the assay was conducted in five different lots of human plasma spiked with celecoxib over the calibration range of 25–2000 ng/ml. The resulting assay precision and accuracy data are presented in Table 1. The intra-day precision of the assay, as measured by the coefficient of variation (% C.V.), was 5.7% or better for all points on the calibration curves. Assay accuracy was found to be within 2.3% of nominal for all standards.

Quality control (QC) samples containing celecoxib at concentrations of 75 ng/ml (low QC), 250 ng/ml (mid QC) and 1750 ng/ml (high QC) were prepared and frozen (−20°C) in 1.5-ml aliquots. Two

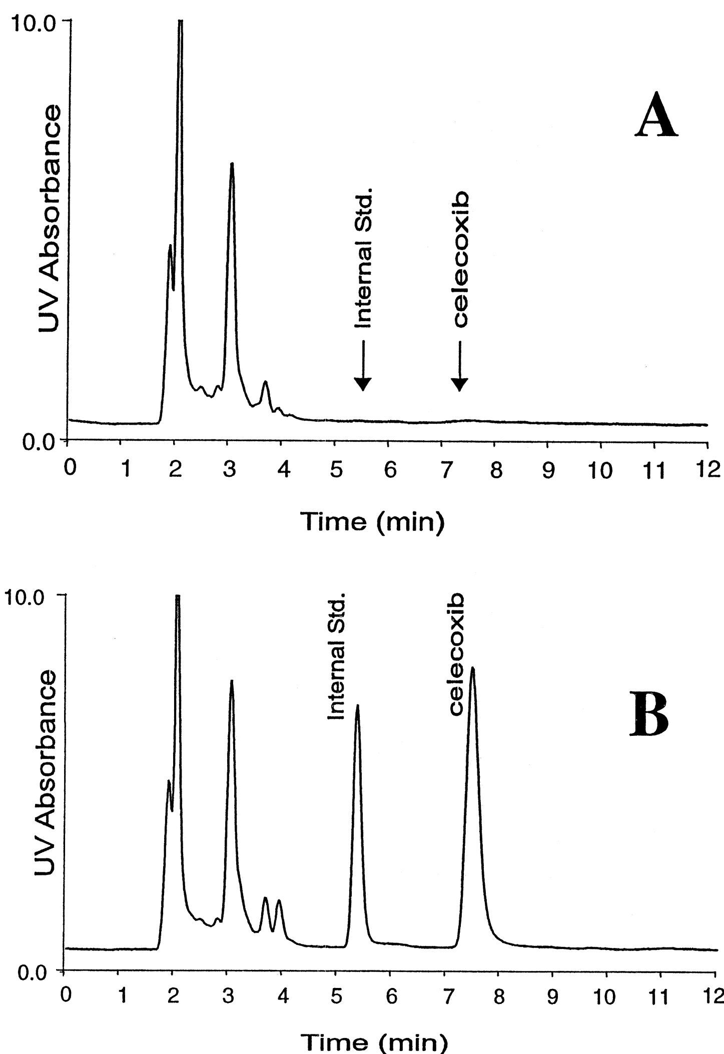

Fig. 3. Chromatograms of plasma samples obtained from a subject receiving celecoxib. (A) Pre-dose sample; (B) sample obtained after receiving 200 mg of celecoxib twice a day for 6 days. The concentration of celecoxib in (B) was determined to be 251 ng/ml.

quality control samples at each concentration were analyzed with each of 11 standard curves over a 15-week period. The results (Table 2) indicate that the between-day variability (RSD) is under 4%. The results also indicate that frozen plasma samples containing celecoxib appear stable for at least 15 weeks.

3.8. Limit of quantification

The limit of quantification (LOQ) of the assay, defined as the lowest concentration that yielded a

Table 2
Inter-day variability of the assay of celecoxib in plasma as assessed by RSDs of low, mid, and high quality control samples

Nominal concentration (ng/ml)	Mean analyzed concentration[a]	RSD (%)
75	73.9	3.2
250	243.2	3.7
1750	1720.2	3.7

[a] $n=11$ over a 15 week period.

within-day RSD of less than 15% and a within-day accuracy of between 85 and 115% of nominal concentration, was 25 ng/ml celecoxib. This LOQ was sufficient to support human studies with the lowest recommended oral dose of celecoxib (200 mg).

3.9. Freeze–thaw stability

Quality control samples ($n=5$ at each concentration) were subjected to three freeze–thaw cycles consisting of a thaw to reach room temperature and then refreezing (−20°C) overnight. These samples together with a set of quality control samples that were not subjected to additional freeze–thaw cycles were then defrosted and analyzed. Results of the analysis are presented in Table 3. For all cases, the celecoxib concentrations for the QC samples that were subjected to additional freeze–thaw cycles were within 2% of those of the control (untreated) samples.

3.10. Clinical sample analysis

The solid-phase extraction/normal-phase HPLC assay for the determination of celecoxib in human plasma was used to determine plasma levels from subjects receiving a single 200-mg dose of celecoxib. The 200-mg dose represents the lowest recommended dose of celecoxib for the treatment of osteoarthritis [2]. Plasma levels from three subjects receiving this dose are shown in Table 4.

Table 3
Assessment of freeze–thaw (F/T) stability of celecoxib in human plasma

Nominal conc. (ng/ml)	Mean found conc. of control samples[a] (ng/ml, $n=5$)	Mean found conc. after three F/T cycles[a] (ng/ml, $n=5$)
75	75.8 (2.8)	75.5(4.0)
250	247.6 (1.7)	242.6(2.9)
1750	1734.3 (0.8)	1723.7(1.6)

[a] Numbers in parentheses are coefficients of variation (% RSD).

Table 4
Plasma concentrations (ng/ml) of celecoxib following single-dose administration of 200 mg to selected volunteers

Subject	Concentration (ng/ml)					
	Time (h)					
	0	2	4	8	12	24
1	nd[a]	1417.4	670.7	236.9	104.1	55.2
2	nd	1425.5	1105.7	471.2	250.6	66.0
3	nd	1069.6	646.6	313.7	156.2	75.0

[a] nd, not detected.

4. Conclusion

An HPLC assay using normal-phase chromatography on a nitrophenyl column has been developed for the determination of celecoxib in human plasma. The method has been found to be precise, accurate and suitable for the analysis of plasma samples collected during human clinical studies with a 200-mg oral dose of celecoxib.

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