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Short communication

Determination of celecoxib in human plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A liquid chromatography–electrospray tandem mass spectrometry method was developed and validated to quantitate celecoxib in human plasma. The assay was based on protein precipitation with methanol and liquid chromatography on a C_{18} column (55 mm × 2 mm, 3 μm), the mobile phase consisted of methanol – 10 mM ammonium acetate (75:25, v/v). Quantification was performed by mass spectrometry in the multiple reaction monitoring mode with negative electrospray ionization at m/z 380 → 316 and 384 → 320 for celecoxib and the internal standard celecoxib-D_4, respectively. The lower limit of quantitation was 7.0 ng/ml using 0.1 ml of plasma and linearity was demonstrated up to 1800 ng/ml. Intra-assay and inter-assay precision expressed by relative standard deviation was less than 4% and inaccuracy did not exceed 6% at all levels. The assay was applied to the analysis of samples from a pharmacokinetic study.

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1. Introduction

Celecoxib (Fig. 1) is the first cyclooxygenase 2 – selective inhibitor introduced into clinical practice. It is approved worldwide for the relief of the signs and symptoms of osteoarthritis, rheumatoid arthritis and ankylosing spondylitis, for the management of acute pain in adults and for the treatment of primary dysmenorrhoea [1].

After administration of 200 mg of celecoxib to healthy volunteers the following pharmacokinetic parameters have been observed: C_{max} 450–800 ng/ml and t_{max} 1.5–3 h. The low solubility of the drug appears to prolong the absorption process making terminal half-life ($t_{1/2}$) determinations more variable. Under fasted conditions, $t_{1/2}$ is approximately 9–11 h. Because of the low aqueous solubility of celecoxib, absolute bioavailability studies have not been conducted [1,2].

Various methods have been published for the determination of celecoxib in biological fluids. They included liquid chromatography (LC) with spectrophotometric detection [3–7], LC–mass spectrometry (LC–MS) [8], LC–inductively coupled plasma mass spectrometry (LC–ICPMS) [9] and LC coupled with tandem mass spectrometry (LC–MS/MS) [10,11]. Tedious extraction procedures, including liquid-liquid extraction [4,5,8,9,11] and solid-phase extraction [3,10] have been used as sample preparation techniques. In two methods [6,7] also protein precipitation was employed, but the less selective spectrophotometric detection at 254 nm necessitated the chromatographic separation of celecoxib and internal standard from endogenous interferences, which resulted in a longer analytical times (6–11 min). The run times were not shorter in all other methods, only in the LC–MS/MS assay [10] the analysis was finished in 4 min, but the extensive sample preparation was required.

The aim of this study was to simplify sample preparation step using protein precipitation and simultaneously to shorten the chromatographic run time with a more selective LC–MS/MS procedure. Also a more suitable internal standard than previously used, isotopically labeled celecoxib, was used to further improve precision and accuracy of the method. These improvements enabled to develop a rapid, simple and sensitive LC–MS/MS method for determination of celecoxib in human plasma. The method was successfully applied to a pharmacokinetic study.

2. Experimental

2.1. Chemicals

Methanol (HPLC gradient grade) was produced by J.T. Baker (Deventer, Holland). Ammonium acetate (puriss. p.a.) was obtained from Fluka (Buchs, Switzerland). Celecoxib was obtained from Zentiva (Prague, Czech Republic) and the internal standard, celecoxib-D_4 was bought from Toronto Research Chemicals (North York, Ontario, Canada).

2.2. Apparatus and conditions

The LC–MS/MS system consisted of the P4000 pump, AS3000 autosampler, TSQ Quantum Discovery Max triple quadrupole mass

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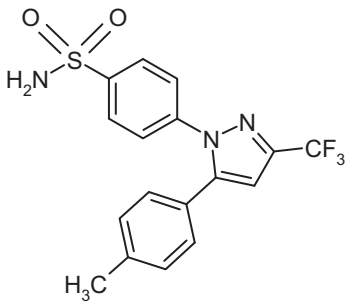

Fig. 1. Chemical structure of celecoxib.

spectrometer with electrospray ion source, data station with Xcalibur software, version 2.0.7 (all from Thermo Fisher Scientific, Waltham, MA, USA). Methanol was used as a washing solution in the autosampler and the injection was performed in a push-loop mode. The temperature of the column oven was 40 °C.

The separation was performed on a Purospher C_{18} column (3 μm, 55 mm × 2 mm, Merck, Darmstadt, Germany) protected with a C_{18} 4 mm × 3 mm precolumn (Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol – 10 mM ammonium acetate (75:25, v/v), the flow-rate was 0.2 ml/min.

The detection of the analytes was carried out using negative electrospray ionization technique and selected reaction monitoring mode to monitor the transitions (precursor → product) *m/z* 380 → 316 and *m/z* 384 → 320 for celecoxib and celecoxib-D_4, respectively. The dwell time was 0.5 s for both analytes and scan width was set to 1.0 *m/z*. Ion spray voltage was set to 3500 V, temperature of the ion transfer capillary was 350 °C. Collision energy was 23 V both for both compounds. The pressure of argon in the collision cell was 1.5 mTorr. The pressure of the sheath gas, sweep gas and auxiliary gas was 60, 6 and 15 arbitrary units, respectively.

2.3. Standards

Stock solution was made by dissolving a suitable amount of celecoxib in methanol. Further standard solutions were obtained by serial dilutions of the stock solution with methanol–water (1:1, v/v) mixture. The standard solutions were stored at −18 °C and were protected from light; they were stable at least 2 weeks under these conditions.

The calibration and quality control plasma samples were prepared by addition of standard solutions to drug-free plasma in volumes not exceeding 3% of the plasma volume.

The solution of the internal standard was obtained by dissolving 1 mg of celecoxib-D_4 in 1 ml of methanol and the precipitation solution containing 100 pg/μl of celecoxib-D_4 was obtained by further diluting this solution with methanol.

2.4. Preparation of the sample

The plasma samples were stored in the freezer at −18 °C and thawed at room temperature before processing of the sample.

One hundred microliters of plasma were pipetted to the polypropylene tube, 600 μl of the precipitation solution containing internal standard was added and the tube was vortex-mixed for 30 s at 2000 rpm. The tube was then centrifuged for 2 min at 2000 × *g* and the supernatant was transferred to an 1.8 ml autosampler vial. Two μl aliquot was injected into the chromatographic system.

2.5. Calibration curves

The concentrations of individual calibration samples were 6.967, 9.929, 27.53, 78.00, 232.9, 628.4 and 1814 ng/ml. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$): the peak area ratio (analyte/internal standard) was plotted vs. the analyte concentration. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

3. Results and discussion

3.1. Method development

Initially the LC with spectrophotometric detection was tried, but many endogenous interfering peaks were present on the chromatograms and it was obvious that the method development would be time consuming. Therefore the further experiments were performed using LC–MS/MS. Both positive and negative ions can be measured using electrospray ion source, the latter were found to yield better signal-to-noise ratio.

Celecoxib was well retained on an octadecylsilica column. The peak of celecoxib had a capacity factor of about 2 (retention time 1.8 min) in the mobile phase containing methanol – 10 mM ammonium acetate (75:25, v/v) and it was well separated from non-retained interferences. The sample solvent with about 85% of methanol was compatible with the mobile phase and the sample after protein precipitation can be injected directly into the column with no peak distortion. The high sensitivity of detection enabled to inject only a small aliquot of the sample (2 μl) and consequently no problems with column clogging were observed.

Typical chromatograms of drug-free plasma (a); spiked plasma at limit of quantitation at 7 ng/ml (b) and plasma from a pharmacokinetic study containing 162.5 ng/ml of celecoxib (c) are shown in Fig. 2. The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks.

Simple protein precipitation with methanol was found sufficient as a preseparation technique. The recovery of celecoxib was nearly quantitative, 97% and 90% at 50 and 1540 ng/ml, respectively.

3.2. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The calibration curve equation is $y = bx + c$, where y represents analyte/internal standard peak area ratio and x represents the analyte concentration in ng/ml. The mean equation (curve coefficients ± standard deviation) of the calibration curve ($N = 11$) obtained from 7 points was $y = 0.001882(\pm 0.000040)\,x - 0.00130(\pm 0.00073)$ (coefficient of determination r^2 was 0.9992).

The limit of quantitation was 7.0 ng/ml. The precision, characterized by the relative standard deviation, was 4.9% and accuracy, defined as the difference between the true and the measured value expressed in percents, was 5.5% at this concentration ($N = 6$).

3.2.1. Intra-assay precision and accuracy

Intra-assay precision of the method is illustrated in Table 1. It was estimated by assaying the quality control samples (low, medium and high concentration) six times in the same analytical run. The precision was better than 4% and the bias was at most ±4% at all levels.

3.2.2. Inter-assay precision and accuracy

Inter-assay precision and accuracy was evaluated by processing a set of calibration and 3 levels of quality control samples on eleven separate runs. The samples were prepared in advance and stored at −18 °C, in each run several samples were processed and analyzed at each level. The respective data are given in Table 1. The precision

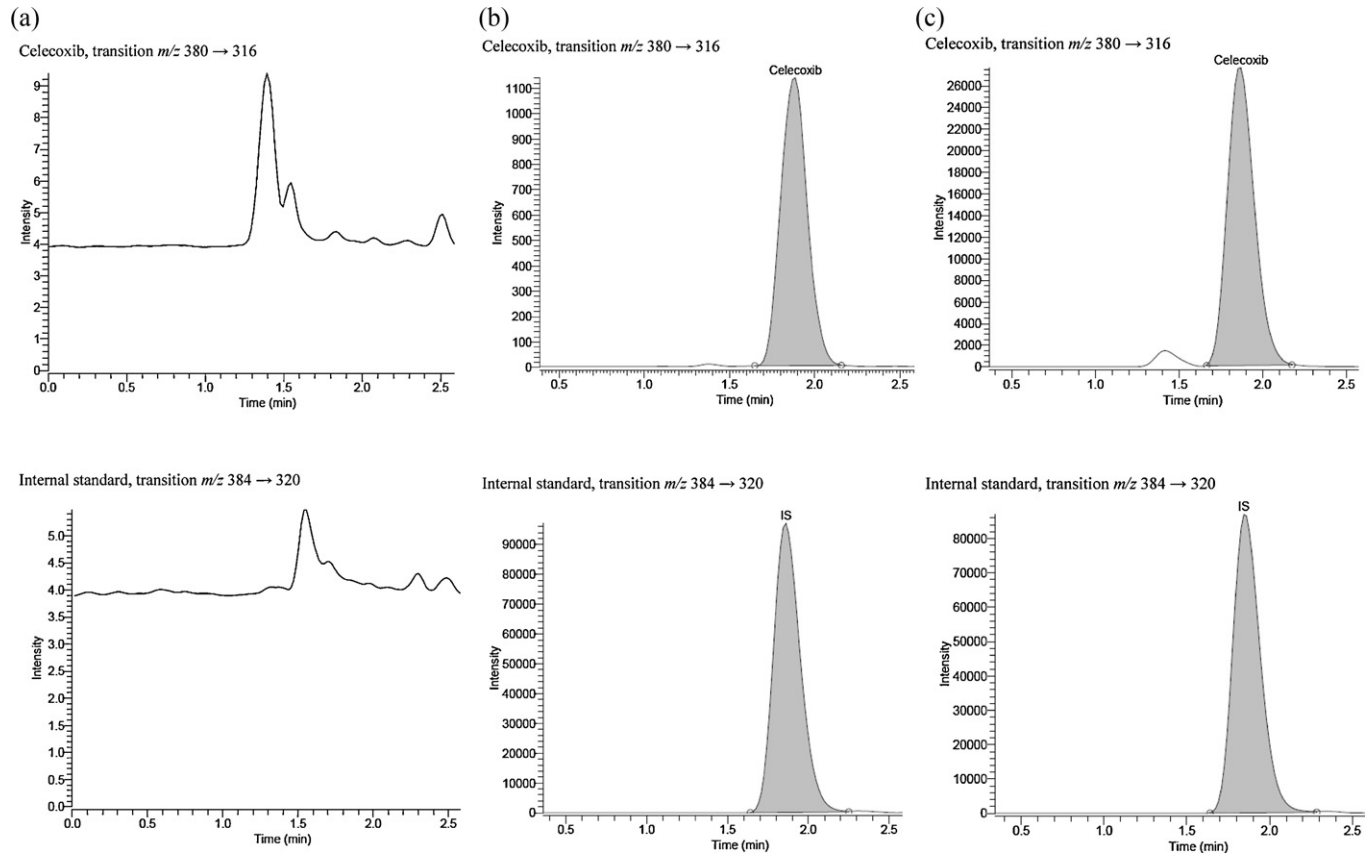

Fig. 2. Chromatograms of (a) drug-free human plasma, (b) spiked plasma at limit of quantitation (7 ng/ml), and (c) plasma sample from a subject 10 h after administration of 200 mg of celecoxib, the concentration of celecoxib was 162.5 ng/ml. The upper panel shows selected reaction monitoring of the transition m/z 380 → 316 (celecoxib) and the lower one the transition m/z 384 → 320 (celecoxib-D_4, internal standard).

Table 1
Precision and accuracy.

N	Concentration (ng/ml)			
	Added	Measured	Bias (%)	RSD (%)
Intra-assay				
6	18.76	18.11	−3.5	3.9
6	157.7	159.5	1.2	2.0
6	1521	1484	−2.4	3.3
Inter-assay				
64	18.76	18.77	0.1	3.7
63	165.9	168.6	1.6	3.2
63	1561	1466	−6.1	3.5

was better than 4% and the inaccuracy did not exceed ±6% at all levels.

3.2.3. Sample stability

Stability was generally concluded if the concentration change was not larger than ±15% compared to freshly prepared samples. The results are shown in Table 2.

3.2.3.1. Freeze and thaw stability. Plasma samples with a low and high concentration of analyte were prepared. The samples were stored at −18 °C and subjected for 3 thaw and freeze cycles. After the third cycle triplicate the samples were processed, analyzed and the results averaged. No significant substance loss during repeated thawing and freezing was observed.

3.2.3.2. Processed sample stability. Two sets of spiked samples with a low and a high concentration of analyte were analyzed and left in the autosampler at ambient temperature. The samples were analyzed using a freshly prepared calibration samples 2 days later. The processed samples were stable at room temperature for this period.

3.2.3.3. Stability of plasma samples. The short-term stability of thawed plasma samples (with a low and high concentration) was studied for period of 24 h at room temperature and ambient light. The long-term stability of frozen plasma samples was examined after one month storage at −18 °C in the dark. The samples were stable under studied conditions.

3.2.4. Matrix-effects

In order to study matrix effects on the ratio of analyte/internal standard peak areas and on the response of individual compounds the following experiment was performed: six different plasma samples (from 5 different subjects and pooled plasma used for preparation of calibrators and quality controls) were spiked with celecoxib (50.4 and 1536 ng/ml for samples with low and high concentration, respectively) and internal standard, processed and analyzed. The relative standard deviation of peak area ratios was 1.9% and 0.6% at low and high concentration, respectively and the relative standard deviation of peak areas of individual compounds was lower than 5%, indicating no significant relative matrix effects, which could negatively influence quantitation results.

3.3. Application to biological samples

The proposed method was applied to the determination of celecoxib in plasma samples from a pharmacokinetic study, which was approved by the local ethics committee. The plasma samples were

Table 2
Stability of celecoxib ($N = 3$).

Matrix	Storage conditions	Concentration added (ng/ml)	Deviation after storage (%)	RSD (%)
Plasma	Three freeze–thaw cycles	26.25	−5.2	1.4
		1521	−2.8	1.3
Plasma	Room temperature, 24 h	26.25	1.4	2.4
		1521	1.4	1.6
Plasma	−18 °C, one month	26.25	−10.5	4.6
		1521	−7.5	3.7
Processed sample	Room temperature, 2 days	26.25	−2.8	4.7
		1521	0.1	4.1

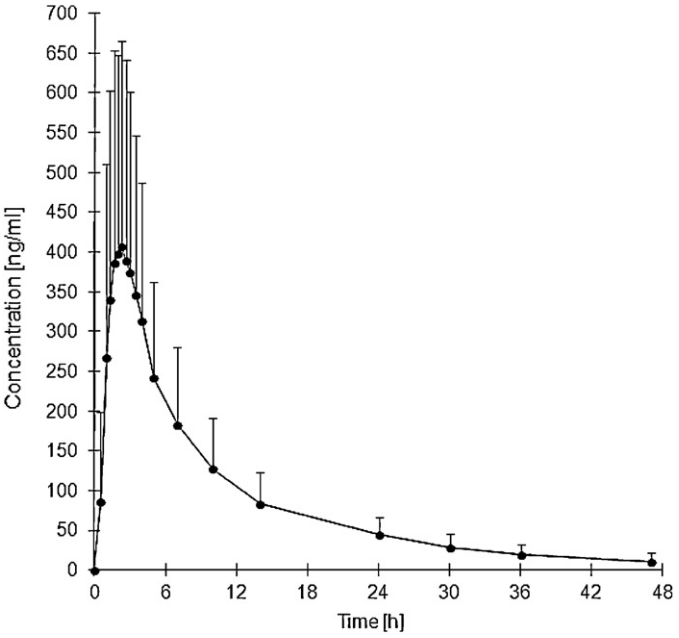

Fig. 3. Mean plasma concentrations (+SD) after a single 200 mg oral dose of celecoxib administered to 44 healthy subjects.

collected following a single oral dose of 200 mg of celecoxib (Celebrex capsules, Pfizer, Germany) administered to 44 healthy male volunteers: mean age of the group was 28 years (range 18–47), mean weight was 79 kg (range 52–95). Fig. 3 shows the mean plasma concentrations of celecoxib; the error bars indicate standard deviations at individual time points.

Plasma levels of celecoxib reached their maximum at 2.4 h (arithmetic mean, range 1.0–5.0 h) after drug administration and thereafter the plasma level declined with an elimination half-time of 11.4 h (range 2.9–44.3 h). The mean C_{max} value was 470 ng/ml (range 170–1380 ng/ml) and the mean area under concentration–time curve (AUC) extrapolated to infinity was 4200 μg h/l (range 2000–10,800 μg h/l). These values were obtained using noncompartmental analysis. The pharmacokinetic parameters are similar to those published earlier [1,2].

4. Conclusions

The validated method allows determination of celecoxib in the 7–1800 ng/ml range. The sample preparation technique is faster and more simple compared to previous methods. The short chromatographic run enables analysis of about 300 samples in one working day. The use of a deuterated internal standard, which is more suitable than those employed in previous methods, contributes to high precision and accuracy of the method. The method was validated according to the guidelines for bioanalytical assays and successfully used for pharmacokinetic studies.

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