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Simple and sensitive method for the determination of celecoxib in human serum by high-performance liquid chromatography with fluorescence detection

Frank Schönberger, Georg Heinkele, Thomas E. Mürdter, Stefanie Brenner, Ulrich Klotz, Ute Hofmann*

Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Auerbachstr. 112, D-70376 Stuttgart, Germany

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Abstract

A simple method is described for the determination of the cyclooxygenase-2 specific inhibitor celecoxib in human serum by HPLC using the demethylated analogue as internal standard. After protein precipitation with acetonitrile, samples were extracted with chloroform. Separation was achieved on a Prontosil C_{18} AQ column (150×3 mm I.D., 3-μm particle size) at a flow-rate of 0.35 ml/min using water–acetonitrile (40:60, v/v) as the mobile phase. Using fluorescence detection with excitation at 240 nm and emission at 380 nm, the limit of quantification was 12.5 ng/ml for a sample size of 0.5 ml of serum. The assay was linear in the concentration range of 12.5–1500 ng/ml and showed good accuracy and reproducibility. At all concentrations intra- and inter-assay variabilities were below 11% with less than 9% error. The method was applied to the determination of celecoxib for pharmacokinetic studies in man.

Keywords: Celecoxib

1. Introduction

Cyclooxygenase (COX) catalyzes the first step in the biosynthesis of prostanoids, the generation of prostaglandin H_2 from arachidonic acid. Two forms of COX exist. The constitutive form (COX-1) is found in healthy tissues and produces physiologically important prostaglandins while the inducible form (COX-2) is predominantly expressed during inflammatory conditions [1,2]. Conventional nonsteroidal antiinflammatory drugs (NSAIDs) inhibit both forms of COX. The assumption, that the antiinflammatory efficacy is due to inhibition of COX-2 while toxic side-effects are mainly caused by inhibition of COX-1 has led to the development of selective COX-2 inhibitors [3].

Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide, Fig. 1) is a COX-2 inhibitor that was recently approved in several countries for treatment of osteoarthritis and rheumatoid arthritis. Available experimental and clinical data show improved gastric tolerance as compared to conventional, non-selective NSAIDs [4,5]. The effects of celecoxib on renal function and

*Corresponding author. Tel.: +49-711-8101-3707; fax: +49-711-859-295.

E-mail address: ute.hofmann@ikp-stuttgart.de (U. Hofmann).

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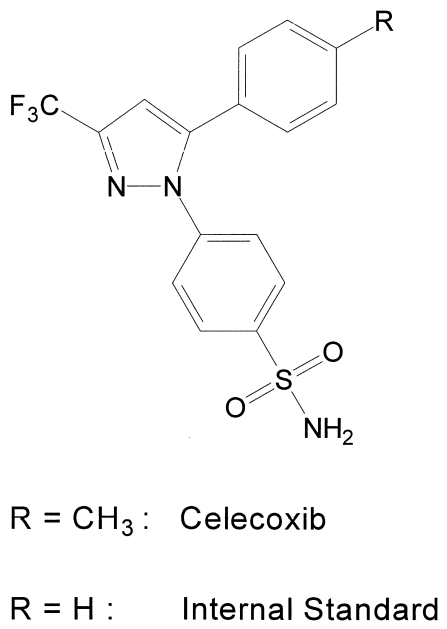

Fig. 1. Structure of celecoxib and the internal standard.

its cardiovascular effects are discussed controversially [6,7].

For further pharmacokinetic/pharmacodynamic studies, e.g. an evaluation of differences in pharmacokinetics between young and elderly patients, we required an assay for the determination of celecoxib in human serum. Till now, only few methods for the quantification of celecoxib have been described utilizing HPLC with UV detection [8,9] or fluorescence detection [10], or liquid chromatography–mass spectrometry [11]. Our intention was to develop a relative simple assay using standard chromatographic equipment. For economic reasons we decided to use liquid–liquid extraction as sample preparation. HPLC is performed isocratically on a reversed-phase column with fluorescence detection. With this method a limit of quantification of 12.5 ng/ml of a sample size of 0.5 ml is achieved, which is comparable or even better than the reported methods.

2. Experimental

2.1. Chemicals

Celecoxib was extracted from celebrex 200 mg capsules (Searle, Chicago, IL, USA). Briefly, the content of two capsules was suspended in water, filtered, and the residue washed with water. After drying over phosphorous pentoxide the residue was suspended in ethyl acetate. The supernatant was evaporated to dryness and crystallised from ethyl acetate–isooctane.

The internal standard 4-[5-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzene-sulfonamide was synthesized according to step 2 of the procedure given by Penning et al. [12]. In brief, a mixture of 16 mmol 4,4,4-trifluoro-1-phenyl-1,3-butadiene and 17.6 mmol 4-sulfonamidophenylhydrazine hydrochloride in 200 ml ethanol was refluxed for 22 h. The solvent was evaporated under reduced pressure. The residue was dissolved in ethylacetate, extracted with water and saline, and the organic solvent evaporated. By crystallisation from hexane–ethyl acetate white needles were obtained. Identity and purity of celecoxib and the internal standard was confirmed by ^{13}C-NMR, ^{1}H-NMR, and elemental analysis. Celecoxib had a sharp melting point of 162 °C.

Chloroform (Uvasol) was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. HPLC instrumentation and chromatographic conditions

The HPLC system consisted of a Rainin Dynamax SD-200 pump (Zinsser, Frankfurt/Main, Germany), a SIL-9A autosampler (Shimadzu, Duisburg, Germany) equipped with a 50-μl loop and a FP-920 fluorescence detector (Jasco, Groß-Umstadt, Germany). Data acquisition and analysis was performed using a personal computer and Hyperdata interfaces and software (Bischoff, Leonberg, Germany). HPLC was performed on a Prontosil C_{18} AQ column (150× 3 mm I.D., 3-μm particle size) at a flow-rate of 0.35 ml/min using water–acetonitrile (40:60, v/v) as the mobile phase. Column temperature was maintained at 15 °C with a Jetstream 2 column thermostat (Bischoff). The detector settings were as follows: excitation 240 nm, emission 380 nm, gain 100, attenuation 64, response 10 s. The run time on the autosampler was set at 16 min.

2.3. Standard solutions

The stock standard solutions of celecoxib and the internal standard (1 mg/ml) were prepared in acetonitrile. Working standard solutions (10 and 1 μg/ml) were obtained by dilution of the stock solutions with acetonitrile.

2.4. Sample extraction

To 0.5 ml of serum 25 μl of internal standard solution (30 ng/μl in acetonitrile) was added. After mixing, proteins were precipitated with 500 μl of saturated sodium chloride and 1 ml of acetonitrile. The samples were extracted with 8 ml of chloroform for 15 min and then centrifuged at 1500 *g* for 15 min. The upper aqueous phase was aspirated and discarded, and the organic phase transferred to a clean tube and evaporated to dryness (N_2). The residue was dissolved in 100 μl of the mobile phase (water–acetonitrile 40:60, v/v) by sonication for 5 min, followed by short vortex mixing. After centrifugation for 15 min the supernatant was transferred to an autosampler vial, aliquots of 10 μl were used for HPLC analysis.

2.5. Standardization and assay validation

2.5.1. Calibration and quality control samples

Calibration samples were prepared every day during ongoing analysis by adding known amounts of celecoxib (12.5, 25, 50, 200, 500 and 1500 ng/ml) to control serum. The samples were submitted to the extraction procedure and HPLC analysis as described above. Standard curves (12.5–1500 ng/ml) were obtained by plotting the peak height ratio of celecoxib to internal standard against celecoxib concentration. The calibration curves were evaluated by linear regression analysis, and concentrations of celecoxib in unknown samples were calculated from the average of the response factors.

To determine assay precision and variability, quality control samples (12.5, 200 and 1500 ng/ml) were prepared by adding the respective amounts of celecoxib to 25 ml of control serum, which was divided into aliquots (1.6 ml) and stored at −20 °C.

2.5.2. Intra-day and inter-day precision and accuracy

Intra-day precision and accuracy were assessed by analysis of three different quality control samples, extracted and analysed 10-fold on a single day. Inter-assay precision and accuracy was determined from the results of the quality control samples, which were extracted and analysed on different days. Precision was expressed as coefficient of variation (% C.V.). Accuracy was calculated as the deviation from the nominal value (% bias). The limit of quantification (LOQ) was determined as the lowest concentration with a coefficient of variation (C.V.) and a bias of $<20\%$.

2.5.3. Extraction recovery

Extraction efficiency was determined for celecoxib at three different concentrations (12.5, 200 and 1500 ng/ml) and for the internal standard at its working concentration (1500 ng/ml). Extraction recoveries were calculated by comparing the peak heights obtained from extracted standards in human serum to those found by direct injection of standard solutions at the same concentration.

2.6. Pharmacokinetic study

The HPLC assay developed was used to investigate the steady state pharmacokinetic parameters of celecoxib in young and elderly volunteers. Celecoxib (200 mg) was administered twice daily for 15 days. Blood samples were drawn before and at different time points until 25 h after administration of the last dose. Blood samples were centrifuged after 30 min and serum was stored at −20 °C until analysed. The study had been approved by the local ethics committee (Landesärztekammer Baden-Württemberg). All volunteers gave their written informed consent prior to participating in the study.

3. Results and discussion

3.1. Extraction and HPLC separation

The present method was developed to determine the concentrations of celecoxib in human serum by an HPLC method using standard equipment. We

found that fluorescence detection has comparable sensitivity and a better selectivity than UV detection. Excitation and emission wavelengths were taken from Ref. [10].

A structural homologue was chosen as internal standard (Fig. 1) as it should possess similar properties with respect to extraction and fluorescence.

For economic reasons we decided to develop a liquid–liquid extraction procedure. Different systems for extraction of celecoxib from serum were compared. An essential step is protein precipitation with acetonitrile and saturated sodium chloride prior to liquid–liquid extraction with chloroform. Acidic extraction as performed from Guirguis et al. [9] resulted in dirtier extracts by appearance, the extraction of interfering peaks was increased without an improvement of the extraction yield. During method development, it was observed, that the quality of the chloroform used was critical for ensuring that clean chromatograms were obtained. Chloroform of analytical grade or for residue analysis from different suppliers already contained disturbing substances. Chloroform for UV-spectroscopy, stabilized with 1% of ethanol, was free from interfering peaks and was used for sample extraction.

The results from the recovery experiments are summarized in Table 1. Average recoveries of celecoxib and the internal standard were found to be 77.9±8.6 or 89.4±8.6% (mean±SD), respectively. For the extraction on Empore disks recoveries of 91–96% are reported [8]. But the recoveries obtained with our method are still sufficient for a sensitive measurement of celecoxib.

Separation of celecoxib and the internal standard from endogenous serum components could be performed on a Prontosil AQ column with satisfactory peak shape when simple mobile-phase mixtures of acetonitrile and water were used. The acetonitrile content of the mobile phase was adjusted for an optimal chromatographic run time. With 60% of acetonitrile the retention times of the internal standard and celecoxib were 9.1 and 11.7 min, respectively. Some late-eluting peaks were observed, but with an autosampler run time of 16 min they were eluted during injection and in the first 6 min of the following run.

Table 1
Extraction recoveries of celecoxib and the internal standard from human serum (mean±SD, $n=3$)

	Concentration (ng/ml)	Recovery (%)
Celecoxib	12.5	80.8±0.2
	200	79.8±13.1
	1500	73.2±8.4
	Average	77.9±8.6
Internal standard	1500	89.4±8.6

3.2. Validation

Specificity of the method was tested with blank human serum. In about 20 samples investigated, no interfering peaks were observed. The chromatograms of a serum blank and of the lowest calibration point (12.5 ng/ml) are shown in Fig. 2A and B. Fig. 2C shows the chromatogram of a serum sample 25 h after the last dosing following a 15-day period with 2×200 mg celecoxib with a concentration of 56 ng/ml celecoxib.

Calibration curves were linear over the entire range measured (12.5–1500 ng/ml), with correlation coefficients (r^2) of 0.9963–1.000. Typical calibration curves (mean±SE, $n=14$) for celecoxib were $y = (0.00678\pm0.00017)x + (0.00796\pm0.00786)$. Accuracy and precision of the method was evaluated with quality control samples as described in Section 2.5.1, the results are given in Table 2. At all concentrations intra- and inter-assay variabilities were below 11% with less than 9% error. The limit of quantification (LOQ) achieved is 12.5 ng/ml, which is better than in the reported LC–MS assay [11] or the HPLC–UV assays [8,9].

The same quality control samples were used for stability testing. Samples were stable for at least 1 year, during storage at −20 °C. During three cycles of freezing and thawing no degradation could be observed. Extracted samples were stable at room temperature for at least 3 days or at 4 °C for at least 2 weeks.

3.3. Assay application

The assay described has been used for the determination of celecoxib in serum samples from volunteers administered celecoxib during clinical studies. The serum concentration–time curves of

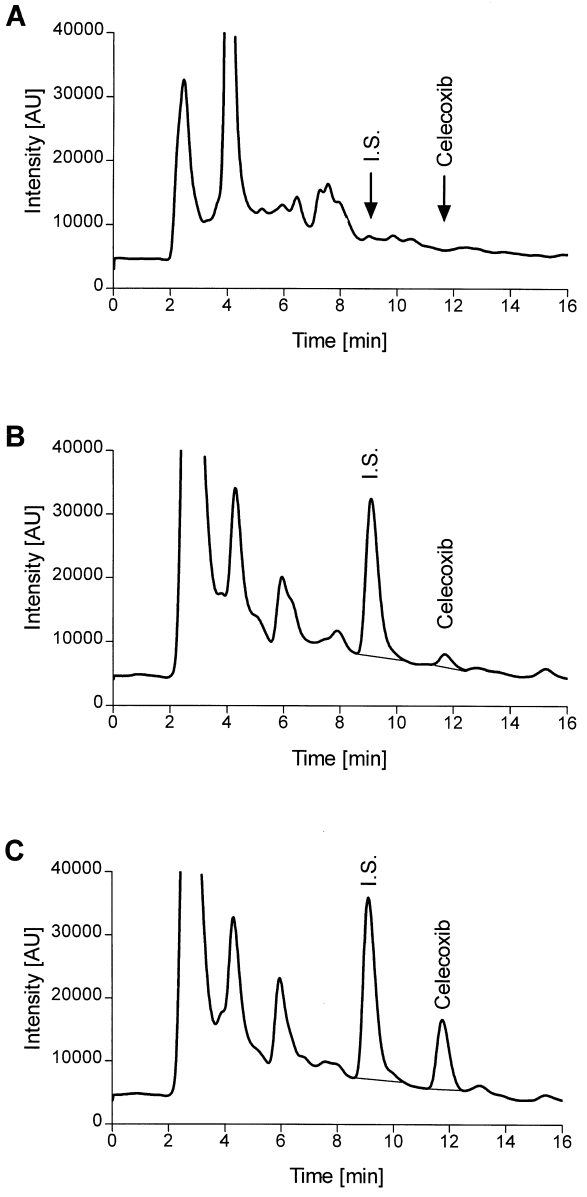

Fig. 2. Representative chromatograms of blank human serum (A), human serum spiked with 12.5 ng/ml celecoxib and 1500 ng/ml internal standard (I.S.) (B), and human serum from a volunteer 25 h after the last dosing (C) following a 15-day treatment with 2×200 mg celecoxib, containing 56 ng/ml celecoxib.

celecoxib in one typical young and one elderly subject following the last dose of a treatment with 200 mg celecoxib orally twice daily for 15 days are shown in Fig. 3. The derived pharmacokinetic parameters of four young and four elderly subjects are summarized in Table 3.

Table 2
Intra- and inter-assay precision and accuracy for the determination of celecoxib in human serum

Concentration added (ng/ml)	*n*	Concentration found (ng/ml)	C.V. (%)	Bias (%)
Intra-assay				
12.5	10	12.4±0.63	5.1	−0.8
200	10	216±9.0	4.2	7.8
1500	10	1480±7.7	7.7	−1.5
Inter-assay				
12.5	15	12.8±1.08	8.5	2.3
200	15	191±15.9	8.3	−4.4
1500	14	1370±148	10.8	−8.7

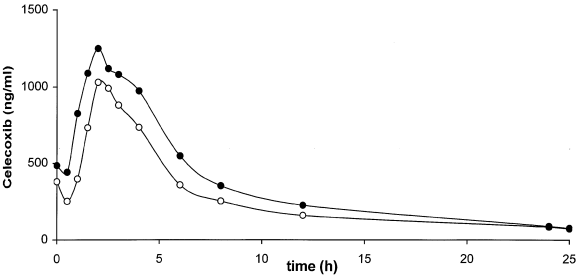

Fig. 3. Serum concentration–time curve of celecoxib in one typical young (●) and elderly (○) subject following the last dosing of a 15-day treatment with 2×200 mg celecoxib.

In conclusion a simple, sensitive and reproducible method has been developed for the determination of celecoxib in human serum using liquid–liquid extraction and HPLC with fluorescence detection, which is suitable for the analysis of celecoxib in pharmacokinetic studies in man.

Table 3
Pharmacokinetic parameters of celecoxib in young and elderly subjects following the last morning dose of a 15-day treatment with 2×200 mg of celecoxib

Parameter[a] (mean±SD)	Young subjects (*n*=4)	Elderly subjects (*n*=4)
$t_{1/2}$ (h)	7.3 (1.4)	9.5 (1.7)
C_{av}^{ss} (ng/ml)	436 (88)	437 (125)
CL_{oral} (ml/min)	659 (147)	677 (188)

[a] Parameters: $t_{1/2}$, terminal half-life; C_{av}^{ss}, mean steady state serum concentration; CL_{oral}, apparent oral clearance.

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