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Determination of celecoxib in human plasma and rat microdialysis samples by liquid chromatography tandem mass spectrometry

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Abstract

Methods for the determination of celecoxib in human plasma and rat microdialysis samples using liquid chromatography tandem mass spectrometry are described. Celecoxib and an internal standard were extracted from plasma by solid-phase extraction with C_{18} cartridges. Thereafter compounds were separated on a short narrow bore RP C_{18} column (30×2 mm). Microdialysis samples did not require extraction and were injected directly using a narrow bore RP C_{18} column (70×2 mm). The detection was by a PE Sciex API 3000 mass spectrometer equipped with a turbo ion spray interface. The compounds were detected in the negative ion mode using the mass transitions m/z 380→316 and m/z 366→302 for celecoxib and internal standard, respectively. The assay was validated for human plasma over a concentration range of 0.25–250 ng/ml using 0.2 ml of sample. The assay for microdialysis samples (50 μl) was validated over a concentration range of 0.5–20 ng/ml. The method was utilised to determine pharmacokinetics of celecoxib in human plasma and in rat spinal cord perfusate.

Keywords: Microdialysis; Determination; Celecoxib

1. Introduction

Celecoxib (4-[5-(4-methansulfonylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] benzenesulfonamid) is a selective cyclooxygenase-2 (COX-2) inhibitor approved for the symptomatic treatment of rheumatoid arthritis and osteoarthritis with an improved side-effect profile in comparison to conventional non-steroidal antiinflammatory drugs (NSAIDs) which inhibit both cyclooxygenases [1].

COX-2 is induced at inflammation sites [2,3] but is also found constitutively in brain, spinal cord, kidney and some other tissues [4–6]. In the rat spinal cord COX-2 has been shown to be upregulated in response to a peripheral nociceptive stimulus. The resulting prostaglandin E_2 (PGE_2) production can be inhibited by NSAIDs [7–9]. Therefore, it is important to assess the distribution of NSAIDs into

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tissues such as spinal cord and to monitor their effects on the release of PGs.

Microdialysis has recently been demonstrated to be a reliable method to evaluate drug and PG levels [10]. In animals microdialysis is an adequate method to determine spinal concentrations of both PGE_2 and celecoxib. Since drug concentrations in dialysates may be low and since the volume of the dialysates is limited to ~50 μl per sampling point, a very sensitive method is required to determine the drug concentrations.

Recently, an HPLC method for the determination of celecoxib in human plasma has been described [11]. The method employs the column switching technique. The run time of this assay is 20 min and the limit of quantification was found to be 25 ng/ml at an injection volume of 65 μl. This assay may be used for routine analysis of plasma samples in clinical studies where the quantification limit of 25 ng/ml may be sufficient.

To assess tissue celecoxib concentrations a more sensitive method, however, is required. Hence we developed an assay using liquid chromatography tandem mass spectrometry (LC–MS–MS). This technique allows improving sensitivity, selectivity and speed of analysis.

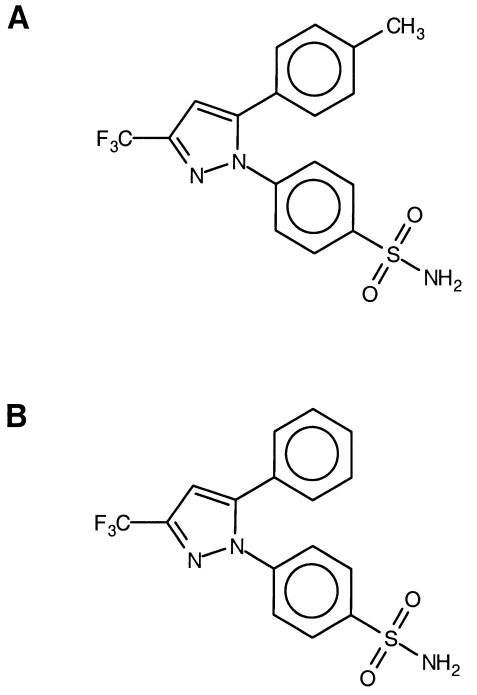

Fig. 1. Chemical structure of celecoxib (A) and the internal standard (I.S.) (B) (4-[5-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide).

2. Experimental

2.1. Materials

Acetonitrile gradient grade was obtained from Merck KgaA (Darmstadt, Germany). The water used was HPLC grade and was obtained from Mallinckrodt Baker (Griesheim, Germany). Potassiumdihydrogenphosphate p.A. and acetic acid were purchased from Riedel-de-Haen (Seelze, Germany). Ammonium acetate was from Fluka (Seelze, Germany). Celecoxib was a gift from Professor W.J. Wechter (Loma Linda University, CA, USA) and the internal standard (4-[5-phenyl-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide, I.S.) was synthesized at the Department of Clinical Pharmacology (Dr Margarethe Fischer-Bosch-Institut, Stuttgart, Germany) according to the procedure described by Penning et al. [12] (Fig. 1). In brief, a mixture of 16 mmol 4,4,4-trifluoro-1-phenyl-1,3-butadione and 17.6 mmol 4-sulfonamidophenylhydrazine hydrochloride (Sigma–Aldrich, Taufkirchen, Germany) in 200 ml ethanol was heated to reflux and stirred for 22 h. Thereafter the solvent was evaporated under reduced pressure. The residue was taken in ethylacetate, washed with water and saline, and the organic solvent was evaporated. By recrystallization from hexane/ethyl acetate (50:50, v/v) white needles were obtained. Identity and purity was confirmed by ^{13}C-NMR, ^{1}H-NMR, and elemental analysis. Celecoxib and I.S. had a purity of >99% as checked by MS and NMR.

Blank human plasma was a gift from the Blutspendedienst Hessen (Deutsches Rotes Kreuz, Frankfurt am Main, Germany).

For microdialysis, catheters were implanted through the dorsal horn of the spinal cord. An artificial cerebrospinal fluid (ACSF) was used as perfusate. It consisted of 115.7 m*M* sodium chloride, 2.6 m*M* potassium chloride, 0.9 m*M* magnesium chloride hexahydrate, 2.1 m*M* sodiumbicarbonate, 2.5 m*M* disodiumhydrogenphosphate dihydrate, 1.3

m*M* calcium chloride dihydrate and 3.5 m*M* D(+)glucose. The solution was sparged with a gas mixture (5% CO_2, 95% O_2) in order to reach a pH of 7.4. All other chemicals were obtained from Fluka (Seelze, Germany) and were HPLC grade.

2.2. Instrumentation

The LC unit consisted of a Jasco DG 1580-53 degasser, a Jasco LG-1580-02 ternary gradient unit, a Jasco PU-1585 pump and a Jasco AS 1550 autosampler (Gross-Umstadt, Germany). The detection was by a PE Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems Langen, Germany) equipped with a turbo ion spray interface. Nitrogen (high purity) was supplied by a Whatman nitrogen generator (Parker Hannifin, Kaarst, Germany).

2.3. LC–MS–MS conditions for plasma samples

Chromatographic separation of extracted plasma samples was performed in isocratic mode with a Nucleosil C_{18} column (30×2.0 mm I.D., 5-μm particle size and 100-Å pore size, Macherey-Nagel, Dueren, Germany). The mobile phase consisted of acetonitrile/water/ammonium hydroxide solution 25% (85:15:0.1, v/v/v). The flow-rate was set at 0.2 ml/min. The injection volume was 10 μl and the run time was 4 min. Celecoxib and I.S. were eluted at ~0.8 min ($k'=0.85$ for both celecoxib and I.S.).

The turbo ion spray interface was operated in the negative ion mode at −3700 V and 400°C and was supplied by an auxiliary gas flow of 4500 ml/min. The nebulizer gas was set at 1.23 l/min, the curtain gas flow was set at 1.08 l/min and the collision gas at 3.7×10^{-6} HPa ($2.92*10^{15}$ molecules/cm^2). Nitrogen was used for all gases.

Quantitation was performed by multiple reaction monitoring (MRM) (dwell time 400 ms) of the deprotonated precursor ion and the related product ion using the internal standard method with peak area ratios and a weighting factor of $1/x$. The mass transitions used were m/z 380→316 (collision energy −32 eV) and m/z 366→302 (collision energy −30 eV) for celecoxib and the internal standard, respectively. The quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.1).

2.4. LC–MS–MS conditions for microdialysis samples

For microdialysis samples a modified chromatographic procedure was used. Microdialysates were separated using a Nucleosil C_{18} column (70×2.0 mm I.D., 5-μm particle size and 100-Å pore size, Macherey-Nagel, Dueren, Germany). The mobile phase consisted of acetonitrile/water/ammonium hydroxide solution 25% (65:35:0.1, v/v/v). The flow-rate was set at 0.2 ml/min. The injection volume was 10 μl and the run time was ~5 min. Celecoxib was eluted at 2.7 min ($k'=4.76$).

The parameters for the mass spectrometer and the turbo ion spray were the same as for plasma samples except for the temperature and the auxiliary gas flow. The turbo ion spray temperature was set at 500°C and was supplied by an auxiliary gas flow of 6000 ml/min.

Quantitation was performed as described above without an internal standard.

2.5. Preparation of stock solution and standards for plasma samples

The stock solution used had a concentration of 1 mg/ml. It was prepared by weighing 10 mg of reference material into a 10-ml volumetric flask and diluting to volume with acetonitrile. The solution was stable for at least 6 months when stored at 4°C. Acetonitrile was spiked with stock solution and subsequently diluted with acetonitrile to obtain working standards in the concentration range of 100, 10, 1 μg/ml. Standards were subsequently diluted with drug free control plasma to obtain concentrations of 0.1–250 ng/ml (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, 250.0 ng/ml).

A stock solution of I.S. was prepared by adding 1 mg reference material into a 10-ml volumetric flask and diluting to volume with acetonitrile. This stock was diluted with acetonitrile/water 50:50, v/v, to prepare a 450-ng/ml solution.

2.6. Preparation of standards for microdialysis samples

Standards for microdialysis samples were prepared using stock solution and working standards as described. Standards were prepared by dilution with ACSF to obtain concentrations in the range of 0.1–20 ng/ml (0.1, 0.2, 0.5, 0.8, 1.0, 2.0, 5.0, 8.0, 10.0, 15.0, 20.0 ng/ml).

2.7. Plasma extraction procedure

Equivalent to the extraction procedure described recently [11], celecoxib and I.S. were extracted by solid-phase extraction. An aliquot of 200 μl of plasma was diluted with 200 μl of 0.1 *M* phosphate buffer (pH 4.0). Then 25 μl of internal standard solution (450 ng/ml) in acetonitrile/water (50:50, v/v) was added resulting in a final I.S. concentration of 50 ng/ml. Chromabond C_{18} (100 mg,1 ml) cartridges (Macherey-Nagel, Dueren, Germany) were placed on a 12-place vacuum manifold equipped with stopcocks at each position. The cartridges were conditioned with 2×1 ml acetonitrile, then washed with 2×1 ml water. After loading the plasma samples cautiously onto the cartridges, they were washed with 2×1 ml water. The cartridges were then dried under reduced pressure (−70 kPa) for at least 5 min. The analyte was eluted with 2×1 ml methylene chloride into a 12-ml polypropylene conical tube (Sarstedt, Nuembrecht, Germany). The solvent was evaporated in a water bath at a temperature of ≤40°C under a gentle stream of dry nitrogen. The residue was reconstituted with 200 μl of mobile phase and transferred to polypropylene microreaction vials (Sarstedt, Nuembrecht, Germany) prior to injection onto the LC–MS–MS system.

Microdialysis samples were injected directly into the LC–MS–MS system. Extraction was not necessary.

2.8. Application of the method

The use of the method for pharmacokinetic studies was demonstrated by analyzing plasma samples from two healthy volunteers after oral administration of an 800-mg single dose. Blood samples were collected up to 42 h. Plasma was stored at −20°C.

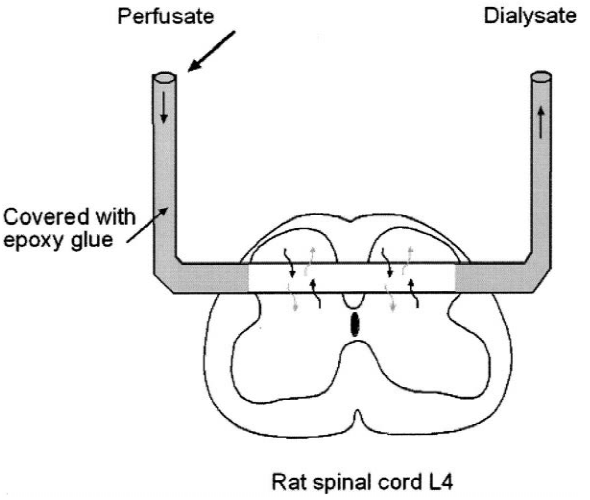

Fig. 2. Microdialysis in the dorsal horns of the lumbar spinal cord of rats.

To assess celecoxib concentrations in the rat spinal cord, microdialysis was employed. Microdialysis catheters (molecular mass cut-off 40 kDa, 0.2-mm I.D., Hospal, Nürnberg, Germany) were implanted transversally through the dorsal horns of the lumbar spinal cord as shown in Fig. 2 [10]. Animals were placed in a free moving chamber and allowed to recover for 24 h. After a baseline sampling for 1.5 h, the rats received a single oral dose of 20 mg/kg. Microdialysis catheters were perfused with ACSF at a flow-rate of 5 μl/min. Samples were collected at 15-min intervals and stored at −40°C.

3. Results and discussion

3.1. Development of the MS–MS conditions

The ion efficiency was tested in the positive and negative ion mode of the turbo ion spray source. To determine the optimal parameter for the mass spectrometer, tuning solutions of 1000 ng/ml of celecoxib and I.S., respectively, were infused. Base peaks for the corresponding masses of celecoxib and I.S. of sufficient intensity for the development of a MS–MS method were obtained for both the positive and negative ion mode.

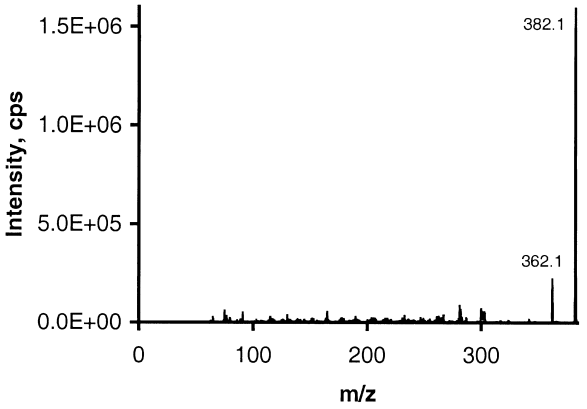

Fig. 3. Positive product ion mass spectrum of the protonated molecule of celecoxib (m/z 380).

The MS–MS system was optimized using the automatic MS–MS tune tool of the Analyst software by infusing a solution of 100 ng/ml of celecoxib and I.S., respectively, at a flow-rate of 10 μl/min. In the positive ion mode for celecoxib only one fragment of a satisfactory intensity was detected with a mass loss of m/z 20. This was considered not specific enough due to the small and hence unspecific mass loss (Fig. 3). By contrast, in the negative ion mode, specific fragmentation resulting in a satisfactory sensitivity was obtained for both substances. Product spectra of celecoxib $[M–H]^-$ m/z 380 and the I.S. $[M–H]^-$ m/z 366 are shown in Fig. 4.

Moreover, the declustering, focussing and entrance potential were optimized by ramping of the respective voltages in the MRM mode infusing a solution of 10 ng/ml of celecoxib.

Different mobile phases containing typical modifiers and buffers (2 m*M* ammonium acetate, ammonium hydroxide solution 25%) and organic solvents (acetonitrile, methanol) were tested for sensitivity. Best results were achieved with solvents containing acetonitrile and 0.1% ammonium hydroxide solution. The basic character of ammonia favours the formation of negatively charged ions and therefore the intensity of the signal.

To increase the sensitivity, flow injection analysis (FIA) was performed at a flow-rate of 0.2 ml/min using a solution of 10 ng/ml of celecoxib. The collision gas density was found to play a significant role for optimization. Best sensitivity was found at 3.7×10^{-6} HPa ($2.92*10^{15}$ molecules/cm^2). These settings were similar for plasma and microdialysis samples.

3.2. Chromatographic system development

Due to the high selectivity of the MRM mode, celecoxib and I.S. were easily distinguished without good chromatographic separation. Therefore a rapid analysis on a short column is possible.

Best conditions for plasma samples resulting in minimal baseline noise and maximum response were found using an isocratic mobile phase which con-

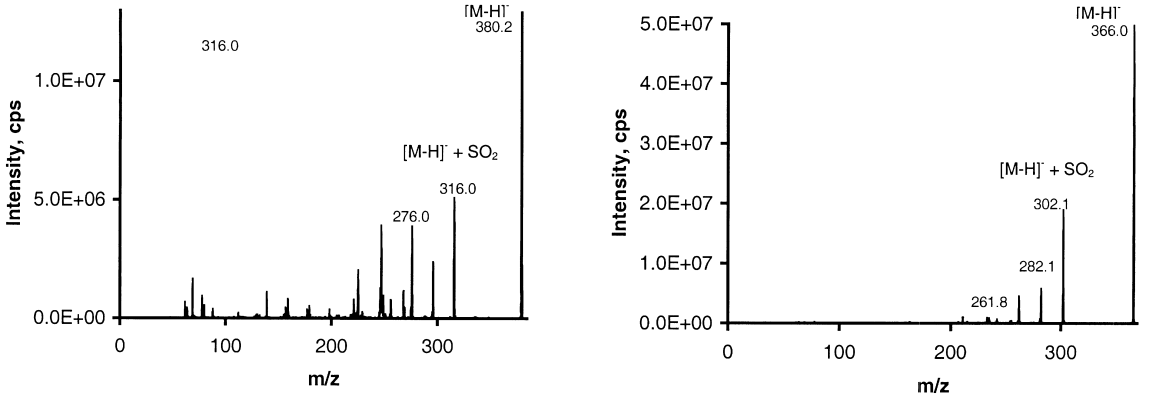

Fig. 4. Negative product ion mass spectra of the deprotonated molecules of celecoxib (A) (m/z 380) and I.S. (B) (m/z 366).

sisted of acetonitrile/water/ammonium hydroxide solution 25% (85:15:0.1, v/v) and a C_{18} narrow bore reversed-phase column (30×2 mm I.D.) for short run times. A flow-rate of 0.2 ml/min resulted in optimal shape of peaks. Smaller flow-rates resulted in higher sensitivity by producing smaller droplets in the ion spray but peaks were broadened resulting in a reduction of sensitivity. Despite the short retention time the run time of 4 min was chosen to allow the column to recalibrate.

Since the ions of the ACSF form clusters in the ion spray resulting in drastic suppression of the signal strength, chromatographic separation of the analyte from the salts was necessary in order to get optimal sensitivity. The sensitivity is related to the proportion of organic solvent in the mobile phase due to a favoured formation of small droplets in the ion spray at higher rates of organic solvent. Therefore, the mobile phase should contain as much organic solvent as possible with sufficient separation of salts and substrate. This was first achieved using a gradient elution. The result was a long runtime due to recalibration of the column. The mobile phase consisting of acetonitrile/water/ammonium hydroxide solution 25% (65:35:0.1, v/v) and a C_{18} narrow bore reversed-phase column (70×2 mm I.D.) worked properly in isocratic mode. Since the percentage of organic solvent had to be reduced, the temperature of the ion spray was increased to 500°C to gain sensitivity.

FIA was used to optimize ion spray settings such as nebulizer gas, curtain gas, ion spray voltage and turbo gas and temperature for the respective mobile phase using a solution of 10 ng/ml celecoxib. Nebulizer gas and ion spray were the most critical parameters for optimization.

To assess the selectivity of the method, extracts of plasma from volunteers and microdialysis samples were analyzed. No crosstalk of celecoxib and I.S. and no interferences with endogenous compounds have been detected in either case. The representative chromatograms of human plasma samples and microdialysates are presented in Figs. 5 and 6.

To assess matrix effects, blank plasma samples

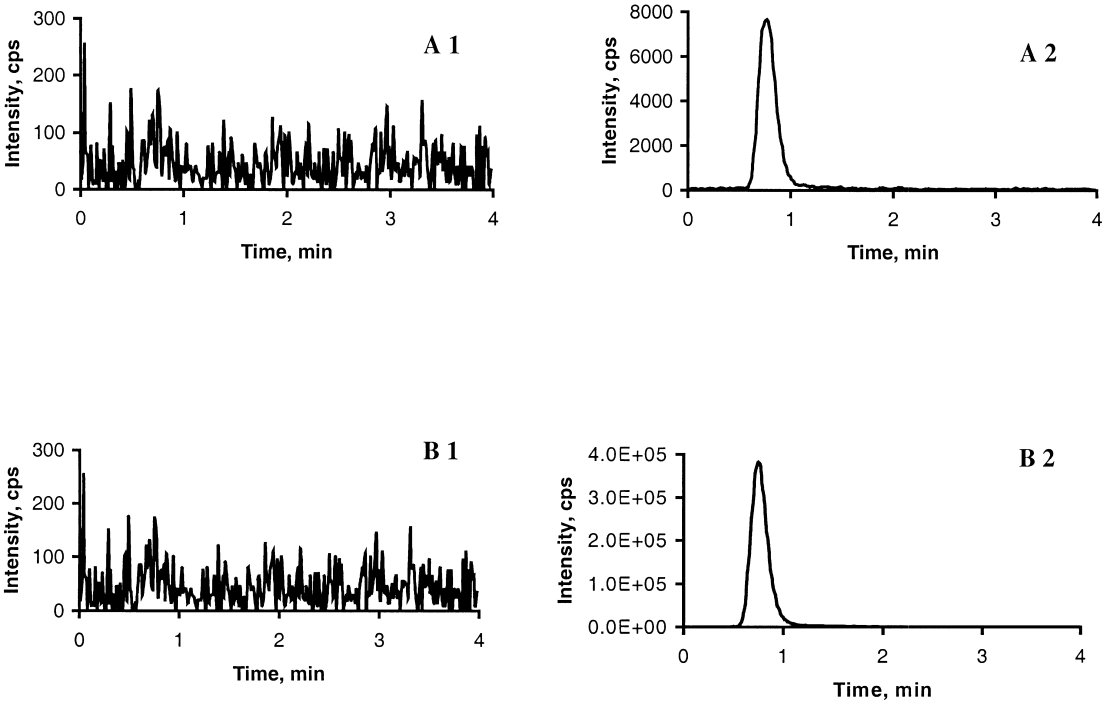

Fig. 5. Representative LC–MS–MS chromatograms of plasma extracts obtained in MRM in negative ionisation mode at m/z 380→316 for celecoxib (chromatograms A) and m/z 366→302 for I.S. (chromatograms B). Chromatograms A1 and B1 represent extracts of blank plasma samples and chromatograms A2 and B2 represent extracts of plasma spiked with 2 ng/ml celecoxib and 50 ng/ml I.S.

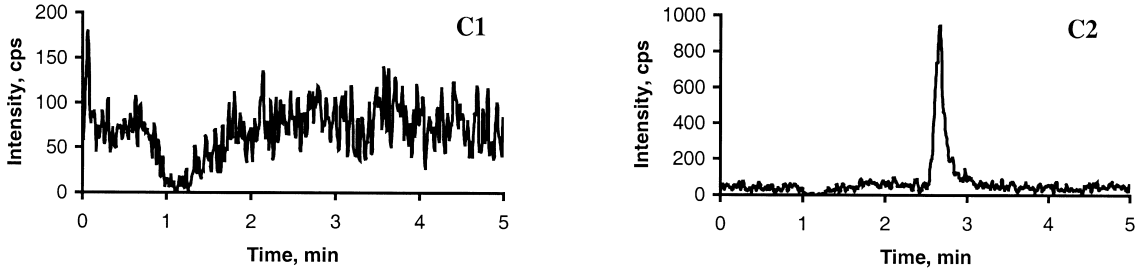

Fig. 6. Representative LC–MS–MS chromatograms of samples in ACSF obtained in MRM in negative ionisation mode at m/z 380→316 for celecoxib. Chromatogram C1 represents ACSF, C2 represents celecoxib (1 ng/ml).

from six different individuals were extracted. The extracts were spiked with celecoxib and I.S. (10 ng/ml). The resulting responses were compared with samples of spiked mobile phase. No significant effects were found between the different plasma samples as compared to the mobile phase samples for celecoxib and I.S., respectively.

The matrix effects of dialysates were assessed comparing the responses of spiked ACSF and spiked mobile phase (10 ng/ml). Although chromatographic separation was achieved, a suppression with a factor of 15 was observed. This effect was reproducible and can be neglected since the standards are also made in the same matrix.

To assess the stability of the used columns regarding the alkaline character of the mobile phase, 240 injections were made using the assay for dialysate samples to evaluate possible degradation of the material. The capacity factor k' and the number of theoretical plates were determined and plotted against the number of injections as shown in Fig. 7. No degradation of the column was observed.

3.3. Extraction procedure development

An extraction method for celecoxib has been previously reported [11]. Nevertheless we tested different extraction procedures using liquid–liquid extraction and solid-phase extraction. Extractions were tested over a pH range of 2–12. For liquid–liquid extraction heptane, ethylacetate, methylene chloride and diisopropyl ether were assessed. In addition different solvent mixtures of the solvents were tested resulting in poor recovery. The best extraction results were achieved using solid-phase extraction. The previously reported procedure was slightly modified in that the eluting step was performed on the vacuum manifold and not using centrifugation. Best results were achieved using

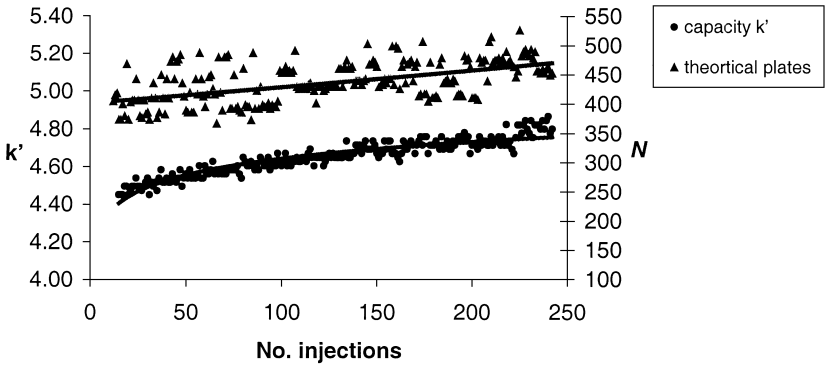

Fig. 7. The lifetime of the column (Nucleosil C_{18}, 70×2 mm, 5 μm, 100 Å) was tested by injecting 10 μl of a celecoxib solution (10 ng/ml) onto the column. Capacity factor k' and number of theoretical plates N were determined using the equations $k' = t_r - t_m/t_m$ (t_r, retention time of the sample peak; t_m, retention time of the unretained peak) for the capacity factor and $N = 5.54 \times (t_r/W_{1/2})^2$ (t_r, retention time; $W_{1/2}$, peak width at half height) for the number of theoretical plates.

methylene chloride in the elution step instead of acetonitrile.

3.4. Extraction recovery of plasma samples

Recovery of the extraction procedure of spiked plasma samples was determined for celecoxib at three different concentrations (1, 20, 100 ng/ml). The recovery of the internal standard was determined at its working concentration of 50 ng/ml. Recoveries were calculated by comparing the absolute peak areas of the extracted samples with diluted standard solutions. Standards were evaporated and reconstituted in the same fashion as extracted samples. The mean recovery for celecoxib was 90%, that for I.S. 85% independent of the concentrations tested.

3.5. Assay validation for plasma samples

Weighted least square regression curves were plotted using the peak area ratio of celecoxib and I.S. versus standard concentrations. The use of weighted least square regression (weighting factor $1/x$) resulted in less than 10% deviation between the nominal standard curve and the experimentally determined standard curve. The calibration curve was found to be linear over the range of 0.25–250 ng/ml.

A total of ten concentrations in six independent series of spiked plasma samples were used to asses the inter-day variability. The resulting assay precision and accuracy data are presented in Table 1. The intra-day precision was assessed using quality control samples with concentrations representing the range of calibration (1, 25, 100 ng/ml). The intra-day precision was determined measuring the coefficient of variation (% C.V.) and was found to be 10, 3 and 4% for the respective concentrations. Assay accuracy was found to be within 10% of nominal values in every case.

Table 1
Precision and accuracy for quantification of celecoxib in plasma

Nominal concentration (ng/ml)	Mean analyzed concentration (ng/ml)±SD ($n=6$)	Coefficient of variation (%)	Accuracy (%)
0.25	0.3±0.02	6.1	110.0
0.5	0.5±0.03	2.8	101.2
1	0.9±0.1	1.1	93.4
2.5	2.4±0.1	2.2	94.7
5	4.9±0.1	2.3	97.1
10	10.1±0.3	3.4	101.1
25	24.9±0.8	3.1	99.7
50	49.3±4.5	9.1	98.6
100	102.0±1.6	1.9	102.0
250	248.8±4.0	1.6	99.4

The limit of quantification was defined as the lowest concentration with a within-day RSD of $\leq$10% and a within-day accuracy between 90 and 110% of nominal concentrations. It was 0.25 ng/ml.

3.6. Assay validation for microdialysis samples

The weighted $(1/x)$ least square calibration curve resulted in less than 10% deviation of standards and was found to be linear over the range of 0.2–20 ng/ml.

Similar to the procedure described for plasma, standard samples of spiked ACSF were prepared in six independent series. The resulting assay precision and accuracy data are presented in Table 2. The intra-day precision was found to be 10% and the accuracy was 8% for all standards. The limit of quantification was 0.5 ng/ml.

3.7. Application of the assay

This LC–MS–MS assay was used to determine the concentrations of celecoxib in human plasma following administration of a single 800-mg dose to two volunteers to span a wide concentration range [13]. Plasma levels of two subjects receiving this dose are presented in Fig. 8. Samples with concentrations above the highest standard were diluted to an appropriate level.

Concentrations of celecoxib in the spinal cord microdialysates of two rats are presented in Fig. 9.

4. Conclusion

A LC–MS–MS assay has been developed for the determination of celecoxib in plasma and microdialysis samples. This LC–MS–MS assay allows a faster analysis of plasma samples and delivers an improved sensitivity for the determination of celecoxib in microdialysis samples. Concentrations

Table 2
Precision and accuracy for quantification of celecoxib in microdialysis samples

Nominal concentration (ng/ml)	Mean analyzed concentration (ng/ml)±SD ($n=6$)	Coefficient of variation (%)	Accuracy (%)
0.5	0.5±0.04	6.5	106.3
0.8	0.8±0.00	0.4	98.6
1.0	0.9±0.09	10.0	94.9
2.0	1.6±0.5	1.5	96.0
5.0	4.6±1.4	8.7	101.4
8.0	7.9±0.4	5.2	98.4
10.0	9.9±0.7	6.7	98.7
15.0	14.9±0.8	5.0	99.1
20.0	20.3±0.9	4.4	101.2

can be measured in the range of 0.25–250 ng/ml in plasma samples and 0.5–20 ng/ml in microdialysis samples. Only 10 μl of sample volume is necessary for the analysis of microdialysis samples.

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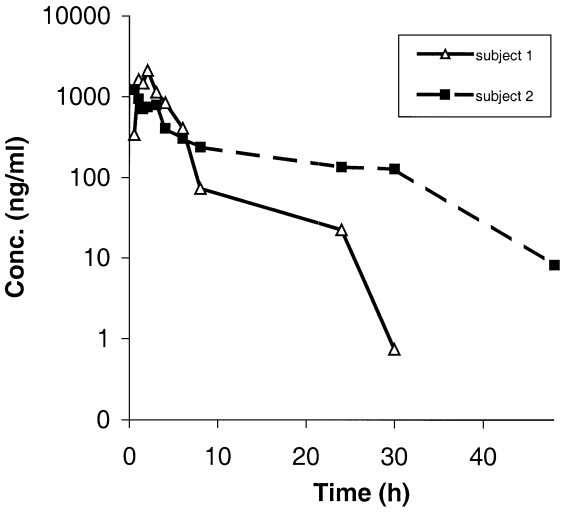

Fig. 8. Concentrations of celecoxib in plasma from subjects receiving an 800-mg dose of celecoxib.

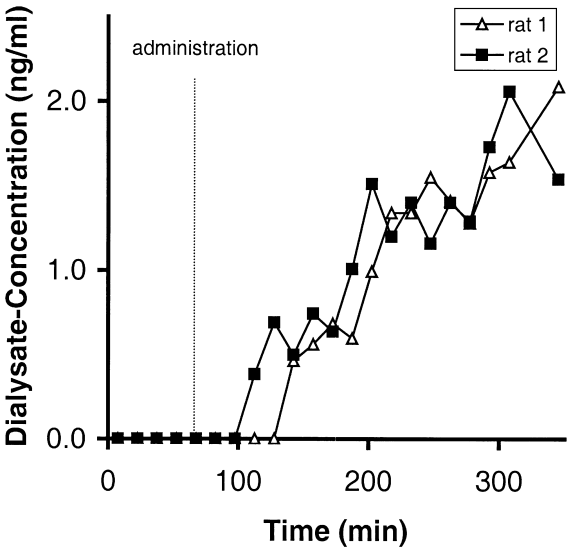

Fig. 9. Concentrations of celecoxib in microdialysis samples from the spinal cord from rats receiving a 20-mg/kg dose of celecoxib.

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