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Journal of Chromatography B, 788 (2003) 309–315
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of etoricoxib in human plasma by liquid chromatography–tandem mass spectrometry with electrospray ionisation

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Received 15 July 2002; received in revised form 12 December 2002; accepted 23 December 2002

Abstract

The validation of a liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the determination of the selective cyclooxygenase-2 inhibitor etoricoxib in human plasma with phenazone as internal standard is described. The plasma samples were extracted by solid-phase extraction using polymer-based cartridges. Chromatography was carried out on a short, narrow bore RP C_{18} column (30×2 mm). Detection was achieved by a Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source working in positive ion mode. The respective mass transitions used for quantification of etoricoxib and phenazone were m/z 359.2→280.2 and m/z 189.0→104.1. The analytical method was validated over the concentration range 0.2–200 ng/ml. The limit of quantification was 0.2 ng/ml. The method is applicable to pharmacokinetic studies in humans.

Keywords: Etoricoxib

1. Introduction

Prostaglandins, formed by cyclooxygenases, are important mediators for a number of physiological processes and pathophysiological conditions, including inflammation and pain [1,2]. Two isoforms of cyclooxygenase have been identified. COX-1 is mainly responsible for the basal physiological production of prostaglandins [3], whilst COX-2 is highly inducible by cytokines or other inflammatory stimuli. Thus, COX-2 plays a major role in the development of pain and inflammation [4,5].

After the approval of two selective cyclooxygenase-2 (COX-2) inhibitors (celecoxib and rofecoxib) for the treatment of osteoarthritis and rheumatoid arthritis, a number of similar substances with increased selectivity are in clinical development.

The main advantage of selective COX-2 inhibitors is that they cause fewer gastrointestinal complications than conventional NSAIDs [6,7] because COX-1 is believed to be responsible for gastro protection.

Etoricoxib {5-chloro-3-(4-methanesulfonyl-

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00034-5

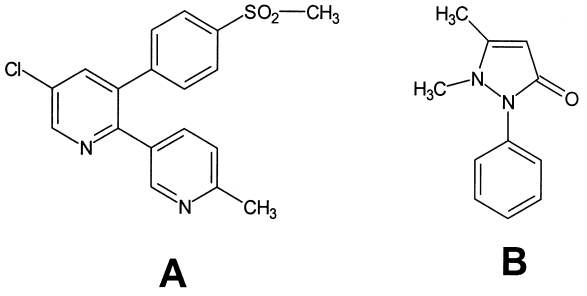

Fig. 1. Chemical structures of etoricoxib (A) and phenazone (B).

phenyl)-6′-methyl-[2,3′]-bipyridinyl} (Fig. 1), which has recently been approved in Mexico and Europe for the treatment of osteoarthritis, has been shown, in vitro, to be the most selective COX-2 inhibitor available thus far [8].

An analytical method for the determination of etoricoxib in human plasma and urine using solid-phase extraction (SPE) and HPLC with photochemical cyclisation and fluorescence detection with a structural analogue as internal standard has been published. The limit of quantification determined by Matthews et al. was 5 ng/ml [9]. A method using LC–MS–MS with atmospheric pressure chemical ionisation (APCI) was published during the development of our work [10]. A stable isotope of etoricoxib was used as internal standard. The runtime of this method was 8 min and it was validated over the concentration range 0.5–250 ng/ml.

In the present report the validation of a LC–MS–MS method is described with phenazone as internal standard, which is not structurally related to etoricoxib, but has the advantage of being commercially available. For the ion source, electrospray ionisation (ESI) was used instead of APCI. The runtime of the present assay was 2.5 min. The assay was validated over the concentration range 0.2–200 ng/ml. This LC–MS–MS assay allows for the determination of etoricoxib with improved speed of analysis and an improved limit of quantification using a commercially available internal standard.

2. Experimental

2.1. Materials

Methanol (HPLC grade) and acetonitrile (gradient grade) were obtained from Merck (Darmstadt, Germany). The water used was HPLC grade and was obtained from Mallinckrodt Baker (Griesheim, Germany). Phenazone (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) was obtained from Sigma–Aldrich (Munich, Germany). Etoricoxib was synthesised by WISTA (Berlin, Germany). The identity and purity were checked by mass spectrometry and ^{1}H-NMR. The purity was >99%. Blank human plasma was a gift from Blutspendedienst Hessen (Courtesy of Dr. R. Henschler, Deutsches Rotes Kreuz, Frankfurt am Main, Germany).

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 equipped with a turbo ion spray interface (Applied Biosystems, Langen, Germany). Nitrogen (high purity) was supplied by a Whatman nitrogen generator (Parker Hannifin, Kaarst, Germany).

2.3. LC–MS–MS conditions for human plasma

Chromatographic separation of extracted plasma samples was performed in isocratic mode with a Nucleodur 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 (90:10, v/v). The flow-rate was set at 0.3 ml/min. The injection volume was 15 μl and the run time was 2.5 min. Etoricoxib and phenazone were eluted in about 0.5 min ($k' = 0.4$ for etoricoxib and phenazone).

The turbo ion spray interface was operated in the positive ion mode at 5400 V and 400 °C and was supplied by an auxiliary gas flow of 4.0 l/min. The nebuliser gas was set at 1.49 l/min (setting 13), the curtain gas flow was set at 1.25 l/min (setting 10) and the collision gas at $5.2 \cdot 10^{-5}$ HPa (setting 9). Nitrogen was used in all cases.

Quantification was performed by multiple reaction monitoring (MRM) of the protonated precursor ion and the related product ion for etoricoxib using the internal standard method with peak area ratios and a

weighting factor of $1/x$. The mass transitions used for etoricoxib were m/z 359.0→280.1 (collision energy 43 eV, dwell time 300 ms) as quantifier and m/z 359.0→279.1 (collision energy 53 eV, dwell time 100 ms) as qualifier. For phenazone, the respective mass transitions were m/z 189.0→104.0 (collision energy 33 eV, dwell time 100 ms) and m/z 189.0→77.1 (collision energy 51 eV, dwell time 100 ms). Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.1).

2.4. Preparation of stock solution and standards for plasma samples

The stock solution of etoricoxib was prepared by weighing 10 mg of reference material into a 10-ml volumetric flask and diluting to volume with acetonitrile, obtaining a concentration of 1 mg/ml. The solution was stable for at least 6 months when stored under light-protected conditions at 4 °C. Drug-free human plasma was spiked with the stock solution and subsequently diluted with acetonitrile–water (50:50, v/v) to obtain standards of concentrations of 100 and 10 μg/ml. These standards were used to prepare working standards in blank plasma by subsequent dilution with drug-free control plasma to obtain concentrations of 2 to 2000 ng/ml (2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, 500.0, 1000.0, 2000.0 ng/ml).

Calibration standards and quality control human plasma samples were prepared by diluting 20 μl of the respective working standard in 180 μl blank plasma to obtain standards in the concentration range 0.2–200 ng/ml (0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0 ng/ml).

2.5. Plasma extraction procedure

Solid-phase extraction was carried out using Oasis HLB (1 ml, 30 mg) cartridges. Prior to the extraction procedure, an aliquot of 200 μl plasma was spiked with 20 μl phenazone solution (500 ng/ml in water), vortexed and centrifuged at 12 000 *g*. The cartridges were placed on a 16-place manifold equipped with stopcocks, conditioned with 1×1 ml methanol and equilibrated with 1×1 ml water. The prepared plasma samples were cautiously loaded onto the cartridges and washed with 1×1 ml water–methanol (95:5, v/v). The cartridges were dried under reduced pressure (−70 kPa) for 5 min. The analyte was eluted with 1×2 ml acetonitrile–ethyl acetate (50:50, v/v). The solvent was evaporated under reduced pressure (−70 kPa) at 45 °C with a centrifugal evaporator (GyroVap, Philip Harris, Leicestershire, UK). The residue was reconstituted in 200 μl mobile phase.

2.6. Application of the method

The assay was applied to plasma samples from a healthy volunteer collected after oral administration of a single dose of 50 mg etoricoxib. Blood samples were collected up to 48 h. The plasma was stored at −20 °C.

3. Results and discussion

3.1. Development of the LC–MS–MS conditions

A solution of 100 ng/ml in acetonitrile–water (80:20, v/v) infused at a flow-rate of 10 μl/min produced a signal of appropriate abundance in the positive ion mode at m/z 359.0 for the protonated precursor ion $[M+H]^+$ using the turbo ion spray. The tuning was processed using the automatic tuning tool of the Analyst software to determine the declustering potential, the focussing potential, the fragmentation pattern, the collision energy and the collision cell exit potential. The same procedure was carried out for the internal standard, phenazone ($[M+H]^+$: m/z 189.0). The resulting fragmentation patterns of etoricoxib and phenazone are shown in Fig. 2. The most abundant fragments for etoricoxib are shown in Fig. 3. These fragments are sufficiently separated by Q3 in unit resolution so that each one can be used for quantification and qualification independently.

The fragments are formed by the secession of moieties of masses of m/z 78.9 and 80.9. The possible sum formulas are CH_3O_2S and CH_4O_2S. The collision energies of these fragments differ by 10 eV, suggesting different fragmentation processes. In one case, the sulfon moiety might separate to leave the protonated fragment of m/z 280.1. SO_2 of the sulfon moiety and CH_4 consisting of the methyl group of the pyridinyl ring and a proton might split

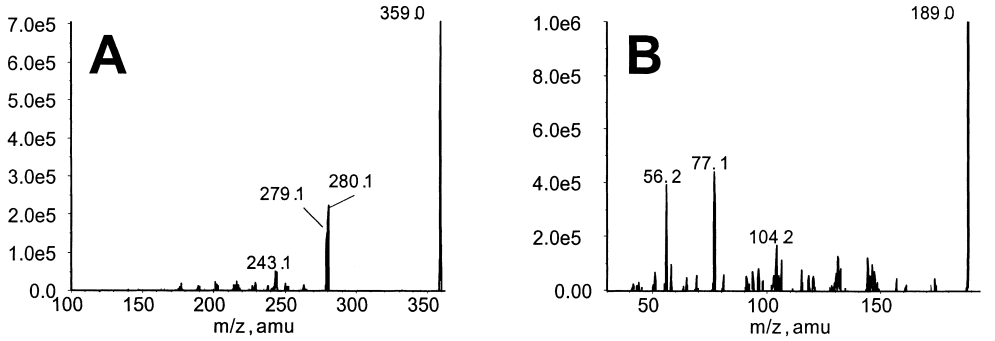

Fig. 2. Product ion scans of protonated molecules of etoricoxib (A) and phenazone (B).

off in a simultaneous process as stable products to give the fragment at m/z 279.1 (see Fig. 3).

Turbo ion spray parameters such as nebuliser gas stream, curtain gas and ion spray voltage were optimised by flow injection analysis (FIA) with a mobile phase flow of 0.2 ml/min. To determine the optimal composition, different mobile phases consisting of methanol–water or acetonitrile–water were tested. Modifiers such as formic acid and ammonium acetate alone or in combination in different concentrations were added. The standard solution of etoricoxib used for FIA was prepared in a particular mobile phase at a concentration of 10 ng/ml. The best signal was achieved using acetonitrile–water (80:20, v/v) without the addition of any modifier.

The tandem mass spectrometer allows the selective detection of substances with varying masses or fragments without chromatographic separation. The development of the chromatographic system was focussed on short retention times and coelution of etoricoxib and phenazone, paying attention to matrix effects as well as good peak shapes. A high proportion of organic solvent was used to coelute both substances [acetonitrile–water (90:10, v/v)]. An increased flow-rate of 0.3 ml/min produced a good peak shape and made a runtime of 2.5 min possible. Shorter run times were not possible with the given setup.

The selectivity was assessed using blank plasma samples. The mass traces of the substances was not affected by endogenous compounds or by each other. The resulting chromatograms for etoricoxib and phenazone and the corresponding blanks from human plasma are shown in Fig. 4.

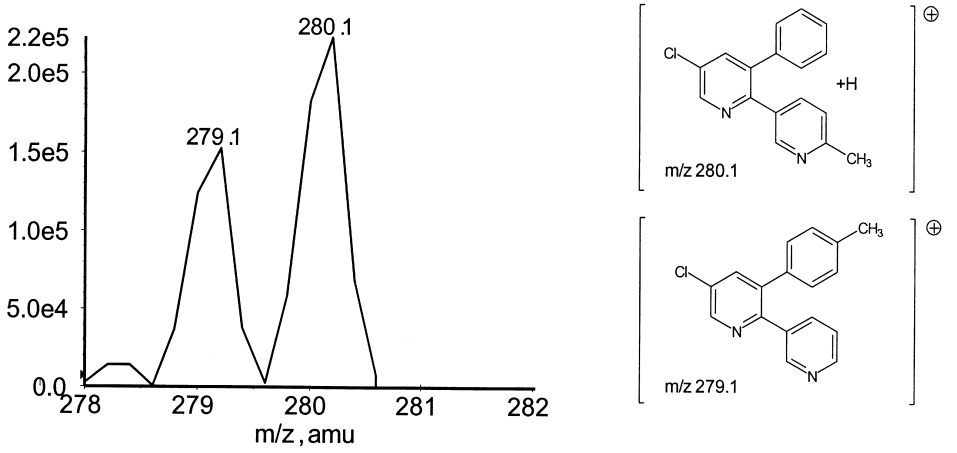

Fig. 3. Fragments of etoricoxib used as quantifier (m/z 280.1) and qualifier (m/z 279.1) and suggested structures of these fragments.

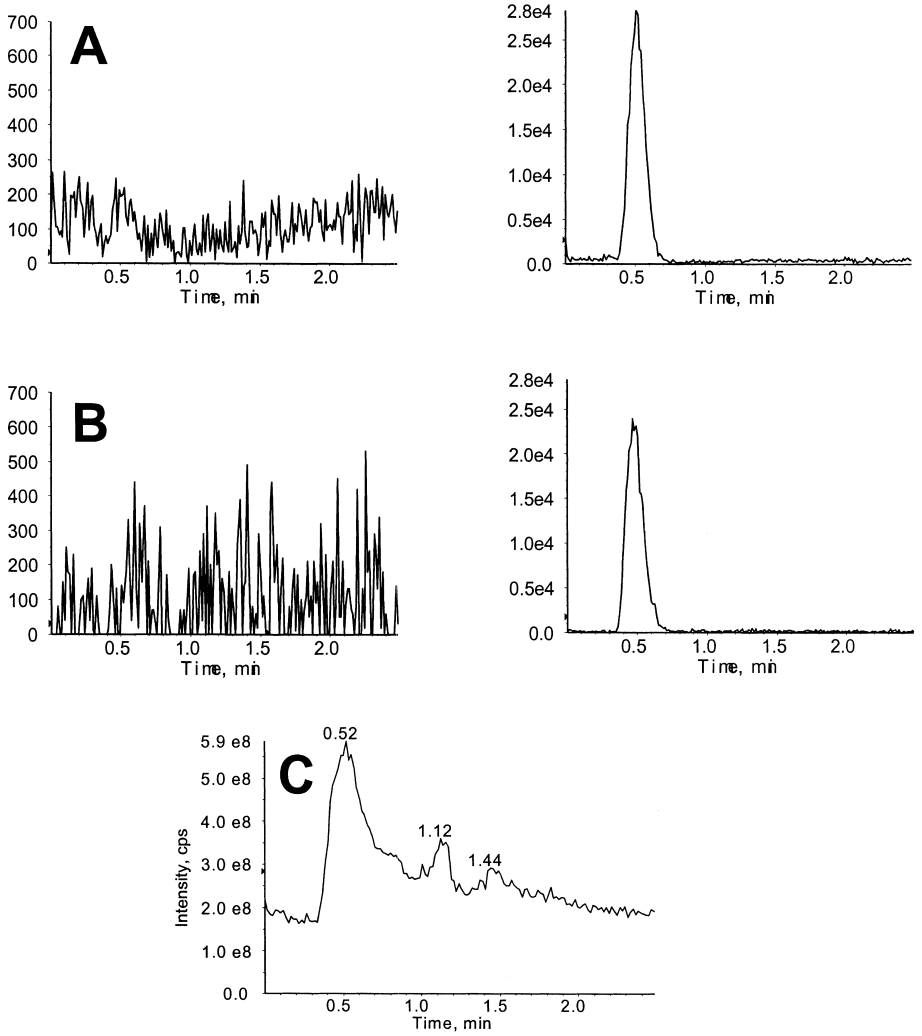

Fig. 4. Representative LC–MS–MS chromatograms of blank and spiked human plasma extracts obtained in MRM in positive ionisation mode at m/z 359→280 for etoricoxib (chromatograms A) and m/z 189→104 for phenazone (chromatograms B). The plasma samples were spiked with 5 ng/ml etoricoxib and 50 ng/ml phenazone. Chromatogram C shows the corresponding total ion current of an extracted plasma sample.

Due to the components of the sample matrix, signal suppression or enhancement may occur. These matrix effects were evaluated by spiking blank plasma extracts with 10 ng/ml etoricoxib or 50 ng/ml phenazone. The resulting chromatograms were compared with chromatograms of pure samples equally concentrated. Six independent plasma lots were used with six samples from each lot. Signal suppression, due to sample matrix effects, of 20% for etoricoxib and phenazone was determined for human plasma. This matrix effect was consistent for all plasma samples tested.

3.2. Internal standard

The best way to cope with sample matrix effects is to use a stable isotope labelled analyte as internal standard. Since no such internal standard is commercially available, an alternative had to be found. Such a substance should match the chromatographic re-

tention, recovery and ionisation properties with the matrix of etoricoxib. Phenazone was found to fulfill these criteria sufficiently. The matrix effects were similar to the matrix effects for etoricoxib. A high proportion of organic solvent was used to achieve the coelution of both substances.

Substances structurally related to etoricoxib such as celecoxib and rofecoxib produced no analytically useful fragments in the positive ion mode (celecoxib) or showed only a weak abundance under the given conditions (rofecoxib).

3.3. Extraction procedure development and recovery

An extraction method for high-throughput analysis has been described previously [9]. A 96-well plate equipped with C_8 material was used to allow automation of the extraction process. Since for routine clinical analysis a high-throughput method is not needed, we were looking for alternative methods. The extraction method should also be suitable for an internal standard that is commercially available without being a stable isotope or a structural isomer of etoricoxib. Solid-phase extraction using a polymer-based material (Oasis HLB) was employed instead of traditional RP C_{18} or C_8 material, since this material is easier to handle and a general method is applicable to a broad range of substances yielding good recoveries. Standard SPE cartridges (1 ml, 30 mg) were used.

The recovery of the extraction procedure for spiked plasma samples was determined for etoricoxib at three different concentrations (1, 10, 100 ng/ml) in human plasma. 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 extracted blank plasma samples spiked with the corresponding concentrations of the analytes. This procedure is necessary to subtract the matrix effects, which can affect the recovery values. The mean recovery for etoricoxib was 90% (±3%) and that for phenazone 85% (±5%), independent of the concentrations tested.

3.4. Assay precision and accuracy

Least square regression curves were plotted using the peak area ratio of etoricoxib and phenazone versus standard concentrations. The regression was weighted by a factor of $1/x$ and resulted in less than 10% deviation between the nominal and the experimentally determined standard curve. The calibration curve was found to be linear over the range 0.2–200 ng/ml.

Table 1
Precision and accuracy for the quantification of etoricoxib in human plasma

Nominal conc. (ng/ml)	Mean analysed conc. (ng/ml)±SD ($n=6$)	C.V. (%)	Accuracy (%)
0.2	0.2±0.02	11	96.5
0.5	0.5±0.06	12	95.1
1	1.0±0.03	2	102.0
2	2.0±0.07	4	102.3
5	4.9±0.2	4	98.6
10	9.9±0.8	8	99.2
20	21.1±0.8	4	105.3
50	50.7±3.7	7	101.2
100	101.6±4.0	4	101.6
200	198.5±3.8	2	99.3

Ten concentrations in six independent series of spiked plasma samples were used to assess the inter-day variability. The resulting assay precision and accuracy data are presented in Table 1. A representative calibration curve is shown in Fig. 5. The intra-day precision was assessed using quality control samples with concentrations representing the range of calibration (1, 10, 100 ng/ml, $n=6$). The intra-day precision was determined by measuring the coefficient of variation (% C.V.) and was found to be 9, 10 and 7% for the respective concentrations.

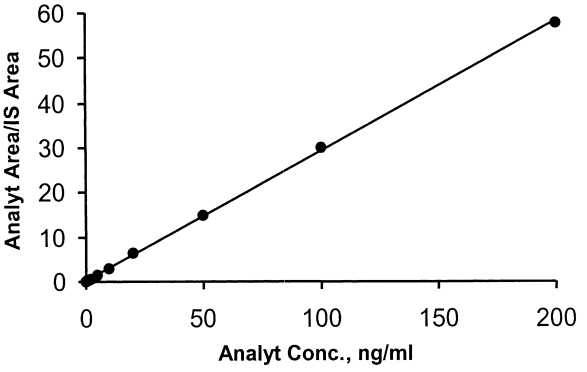

Fig. 5. Representative calibration curve with $1/x$ weighting of the etoricoxib concentration versus the etoricoxib peak area/phenazon peak area ($y = 0.241x + 0.39$).

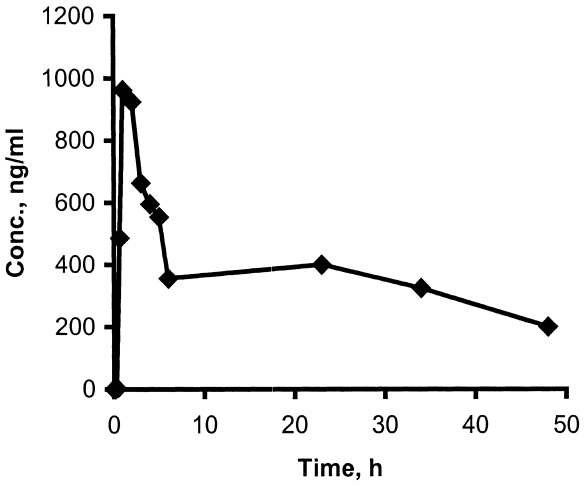

Fig. 6. Concentration versus time profile over 48 h of etoricoxib in human plasma from a subject receiving a single 50 mg dose of etoricoxib.

Assay accuracy was found to be within at least 12% of the nominal values in every case.

The limit of quantification was defined as the lowest concentration with a within-day RSD of 15% and a within-day accuracy of between 85 and 115% of the nominal concentrations. The limit of quantification was 0.2 ng/ml.

3.5. Application of the assay

The concentration versus time profile of a subject receiving a single oral dose of 50 mg etoricoxib is presented in Fig. 6. Samples with concentrations above the highest standard were diluted to an appropriate level.

4. Conclusion

The LC–MS–MS assay described in the present work used an electrospray ion source (turbo ion spray). The tandem mass spectrometry technique permits the selective determination of substances without detailed chromatographic separation and thus a fast analysis. The commercially available substance phenazone worked well as an internal standard for human plasma samples. It was important to coelute etoricoxib and phenazone to compensate for possible matrix effects due to the short retention time. This was achieved by using a short narrow bore column (30×2 mm) and a mobile phase containing a high proportion of organic solvent [acetonitrile–water (90:10, v/v)]. Thus the run time was improved to 2.5 min at a flow-rate of 0.3 ml/min. The sensitivity of the assay was improved to the concentration range between 0.2 and 200 ng/ml.

Acknowledgements

We thank Karin Hehlert and Maic Seegel for excellent technical assistance. This project was supported by the Deutsche Forschungsgemeinschaft (DFG).

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