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Selective and rapid liquid chromatography–mass spectrometry method for the quantification of rofecoxib in pharmacokinetic studies with humans

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Abstract

An easy, rapid and selective method for the determination of rofecoxib in human plasma is presented. The analytical technique is based on reversed-phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionisation mass spectrometry (Finnigan Mat LCQ ion trap). The retention time of rofecoxib was 1.2 min. The method has been validated over a linear range from 1 to 500 μg/l using celecoxib as internal standard. After validation, the method was used to study the pharmacokinetic profile of rofecoxib in 12 healthy volunteers after administration of a single oral dose (12.5 mg). The presented method was sufficient to cover more than 95% of the area under the curve. The pharmacokinetic characteristics (mean±SD) were t_{max}: 2.4±1.0 h, c_{max}: 147±34 μg/l, AUC_{∞}: 2038±581 μg h/l and $t_{1/2}$: 11.3±2.1 h.

Keywords: Rofecoxib

1. Introduction

Rofecoxib (Fig. 1a) is a new nonsteroidal anti-inflammatory agent with selective cyclooxygenase type 2 (COX-2) inhibitory activity. At therapeutic concentrations in humans, rofecoxib does not inhibit the cyclooxygenase-1 (COX-1) [1].

Nonsteroidal anti-inflammatory agents in clinical use inhibit both isoforms of the cyclooxygenase (COX-1 and COX-2) to varying degrees. COX-1 is the constitutive isoform of the enzyme, and its inhibition appears responsible for adverse gastroin-

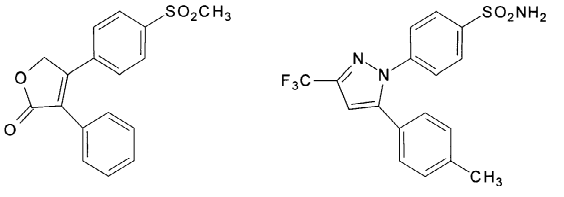

Fig. 1. Chemical structures of rofecoxib (a) and celecoxib (internal standard, b).

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testinal and renal effects, and the anti-platelet activity. In contrast, inhibition of the inducible COX-2 isoform is mainly responsible for anti-inflammatory and analgesic properties. Selective COX-2 inhibitors are claimed to provide analgesic/anti-inflammatory effects comparable to conventional nonsteroidal anti-inflammatory agents, but with a reduced propensity for adverse effects (for review see Ref. [2]).

Since rofecoxib is an increasingly prescribed drug, there is a growing interest in its quantification. A high-performance liquid chromatography (HPLC) method using post-column photochemical derivatisation and fluorescence detection has been described in the literature [3]. As this assay represents an involved and time consuming approach, we tried to use a mass spectrometric technique for the determination of rofecoxib in order to overcome these limitations. In the literature, only one HPLC method with tandem mass spectrometric detection has been described, where Chavez-Eng et al. [4] used an API III Plus triple quadrupole tandem mass spectrometer (PE-Sciex) in the positive and negative ion modes. The limits of quantification (LOQ) are reported to be 10 and 0.1 μg/l, respectively.

In contrast, we used a Finnigan Mat LCQ ion trap spectrometer for the determination of rofecoxib. In the negative ion mode we recorded the same daughters found by Chavez-Eng et al. However, the performance of the assay was much lower and not satisfactory. In the positive ion mode we detected another main daughter, and the performance was suitable to achieve an LOQ of 1 μg/l for the determination of rofecoxib. Finally, a method has been established for plasma specimen sensitive enough to monitor rofecoxib after oral administration of the lowest daily dose recommended (12.5 mg).

2. Experimental

2.1. Reagents and solutions

Rofecoxib and celecoxib were kindly supplied by G. Geisslinger (Center of Pharmacology, Institute of Clinical Pharmacology, Frankfurt a.M., Germany). All other reagents were purchased from Merck (Darmstadt, Germany) in analytical grade. Methanol and acetonitrile were of HPLC grade.

The stock solution of rofecoxib (50 mg/l) was prepared with acetonitrile and stored at −80°C for no longer than 2 months.

Celecoxib was used as the internal standard solubilised in acetonitrile–water (50:50, v/v; stock solution: 1 mg/l). Fig. 1b shows the structural formula of the internal standard.

All standard solutions were found to be stable at −80°C for at least 2 months and for a minimum of 2 weeks when stored protected from light at ambient temperature.

2.2. Sample preparation

An aliquot of plasma sample (1.00 ml), 100 μl acetonitrile, and 75 μl internal standard solution were vortex-mixed for approximately 1 min. Afterwards, 1 ml 0.1 *M* sodium acetate buffer (pH 5) and 4 ml dichloromethane–hexane (50:50, v/v) were added. The tubes were capped, agitated in an overhead shaker for 10 min (45 min^{-1}) and centrifuged at 4000 *g* for 10 min. The organic layer (3.5 ml) was transferred to another tube. The solvent was evaporated under a stream of nitrogen at 40°C. The resulting residue was reconstituted in 140 μl of mobile phase and aliquots of 100 μl injected onto the column. Plasma calibration standards were prepared in the same manner, the 100 μl acetonitrile containing rofecoxib to achieve different final concentrations (1, 5, 10, 25, 50, 100, 250 and 500 μg/l).

2.3. Instrumentation

The HPLC system consisted of a PU-1585 pump and an AS-1550 auto-injector (Jasco, Groβ-Umstadt/Germany). Masses were acquired on a Finnigan MAT LCQ ion trap spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) interface (Thermoquest, Egelsbach, Germany) and connected to a personal computer running the standard software Navigator (1.2).

2.4. Liquid chromatography–mass spectrometry

HPLC was carried out isocratically at ambient temperature using a Nucleosil C_8 guard column (120-5, 11 mm×2 mm; Macherey & Nagel, Düren, Germany), and an eluent comprised of methanol–

water (50:50, v/v) and 1% acetic acid at a flow-rate of 200 μl/min, the outlet coupled to the mass spectrometer's APCI source.

The vaporiser temperature was set to 450°C and N_2 was applied as the sheath and auxiliary gas at flow-rates of 80 and 20 (arbitrary units), respectively.

The heated capillary was maintained at 140°C. Mass analysis was performed at unit resolution in the positive ion mode with the corona discharge current set to 5 μA, and the potentials of both, tube lens and capillary to 15 V. The ion trap was operated in the on-line tandem mass spectrometry (MS^2) mode and the transitions of rofecoxib [parent m/z 315.4, CID (collision-induced dissociation) energy 20%, product m/z 297.0] and celecoxib (382.4, 28%, 362.0) were followed by selected reaction monitoring (SRM) with a maximum automatic gain control (AGC) ion storage time of 500 ms and a mass isolation width of 2 u; three microscans were collected per spectrum.

The peak area ratios of the analyte and the internal standard were calculated. For the validation of the method as well as quantitative analysis of the substrate the relevant principles [5] were taken into account. The standard functions were plotted with concentrations 0, 1, 5, 10, 25, 50, 100, 250, 500 μg/l. The intra-day repeatability of the method was tested by multiple analysis of individual human plasma samples on the same day. Inter-day reproducibility was assessed on 3 different days. The recovery of rofecoxib was determined at 1.0, 25 and 500 μg/l by comparing the peak area after extraction of human plasma standards with the peak area obtained from injection of the same amount of rofecoxib aqueous standards.

2.5. Patients and collection of plasma

A single oral dose of rofecoxib (Vioxx, 12.5 mg tablet, MSD) was administered to 12 healthy human volunteers (mass: 78.8±8.5 kg, age: 27.6±2.6 years, Table 2). EDTA blood samples (5 ml) were taken before and 1, 2, 4, 8, 12, 24, 32, 48, 56 and 72 h after administration and centrifuged immediately. The plasma samples were stored at −80°C for no longer than 2 months.

Informed consent was obtained from all study participants according to the Declaration of Helsinki.

2.6. Pharmacokinetic methods

Plasma concentration–time curves were evaluated by non-compartmental analysis using TOPFIT 2.0 [6]. The apparent half-life, $t_{1/2,\lambda_z}$, was calculated as $\ln(2)/\lambda_z$, where λ_z denotes the time constant of the terminal slope. The area under the plasma concentration–time curve after an oral dose (AUC_{or}) was calculated using the linear trapezoidal rule. The extrapolated AUC_∞ after the last observed plasma concentration was obtained by dividing this plasma concentration by λ_z.

3. Results

The described method yields meaningful results for the quantitative analysis of rofecoxib within 4 min (Figs. 2 and 3). The retention times of rofecoxib and the internal standard celecoxib were 1.2 and 2.3 min, respectively. When analysing a plasma specimen without the addition of rofecoxib or internal standard the chromatograms contained no spurious signals in the relevant portions of the trace (Fig. 4). Chromatograms obtained from 20 different human plasma samples showed that the method is adequately specific. Furthermore, plasma samples spiked with common analgesics (diclofenac, flurbiprofen, ibuprofen, indomethacin, acetaminophen, acetylsalicylic acid, ketoprofen, phenylbutazone) were analysed. None of these substances interfered with the method for quantification of rofecoxib.

Three separate analytical series analysed in duplicate were used to verify linearity of the calibration curve ($y=51x-0.43$) for the relevant range from 1 to 500 μg/l in human plasma. The correlation coefficients (r^2) were greater than 0.995.

Assay checks continued for 2 months demonstrated that the stability of rofecoxib in human plasma at three concentrations (1, 25, 500 μg/l, $n=5$ in each case), and the standard solution of rofecoxib (50 mg/l acetonitrile) stored at −80°C, including two freeze–thaw cycles, was adequate (difference between measured value before and after freeze–thaw cycles <5% in each case).

The intra- and inter-day assay precision and accuracy for low, medium and high concentrations of

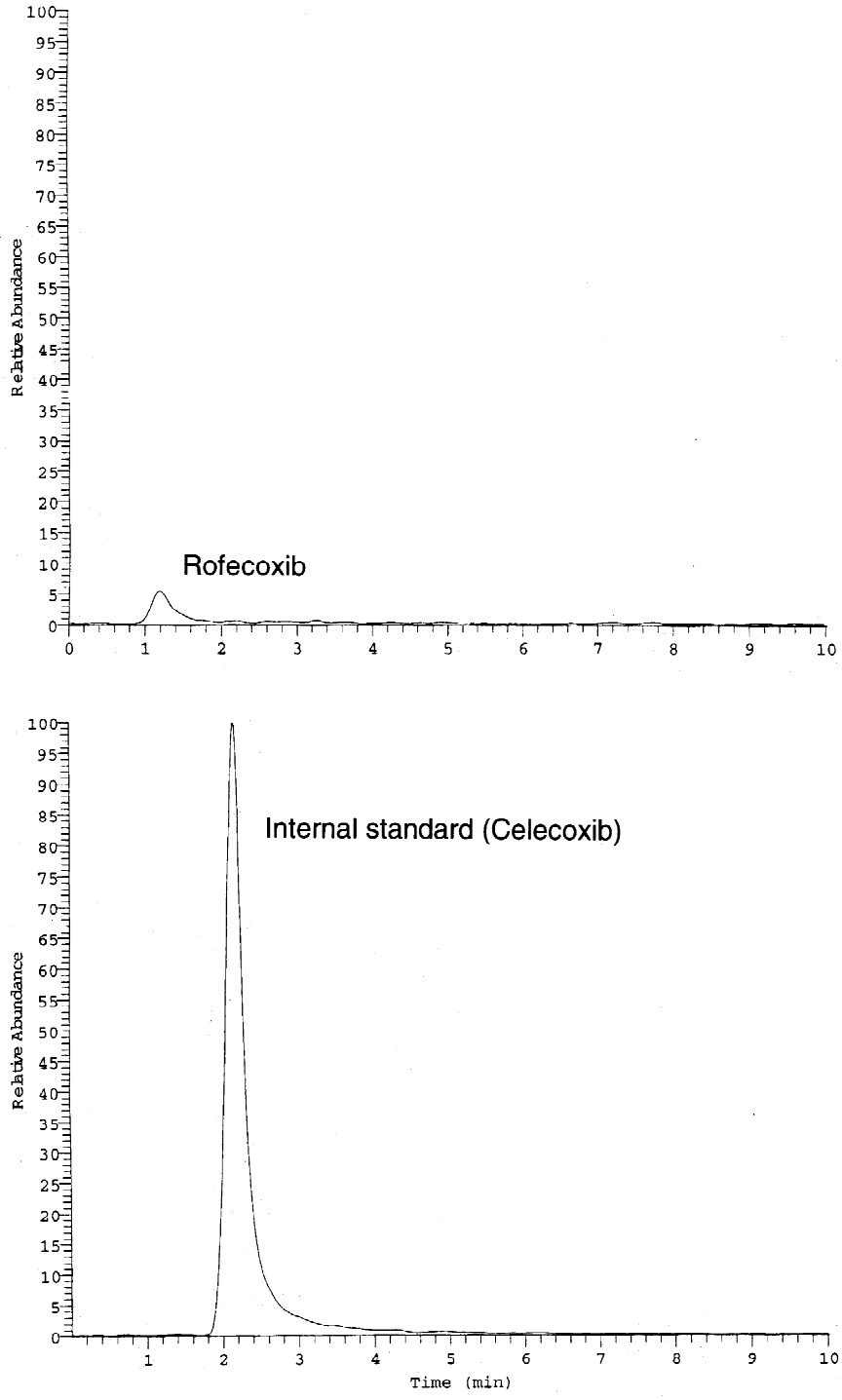

Fig. 2. Representative SRM chromatogram of human plasma spiked with 1 μg/l rofecoxib (LOQ) and internal standard celecoxib (75 μg/l).

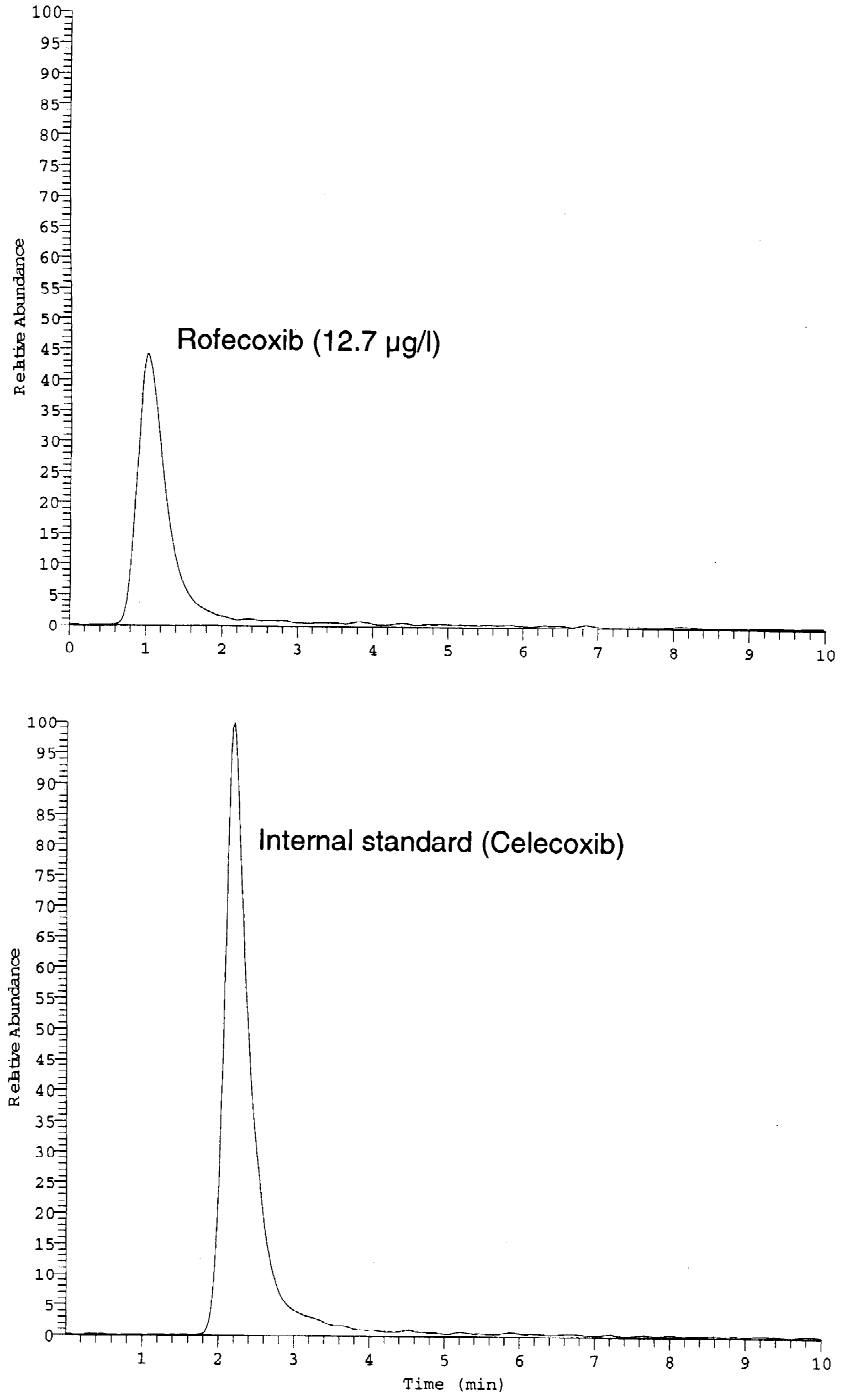

Fig. 3. SRM chromatogram of rofecoxib and internal standard in human plasma sample 48 h after oral administration of 12.5 mg.

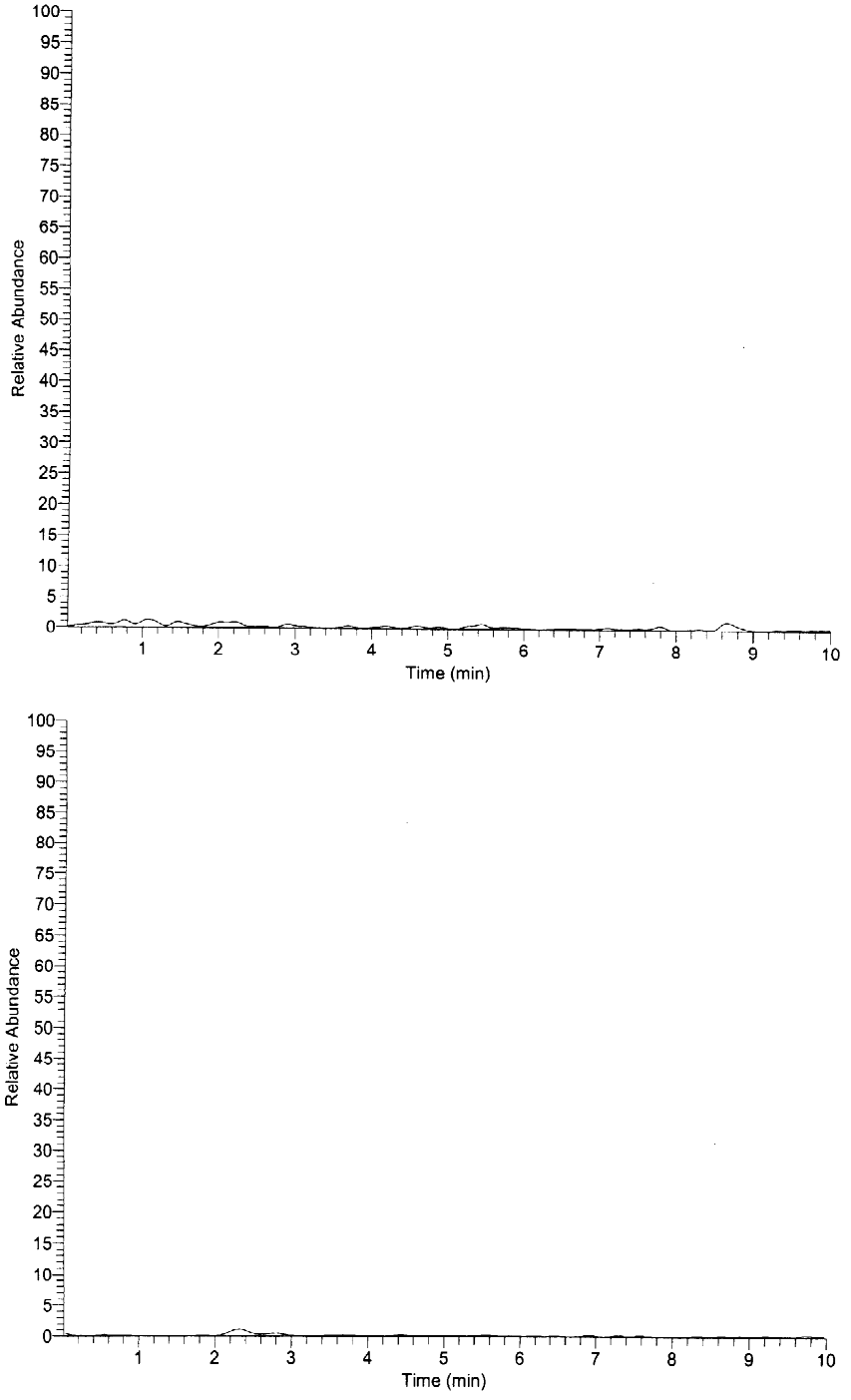

Fig. 4. Representative SRM chromatogram of human plasma lacking both, rofecoxib and internal standard.

Table 1
Precision (relative standard deviation, RSD) and accuracy for low, medium and high concentrations of rofecoxib in human plasma

Added concentration (μg/l)	Intra-day (n=5)			Inter-day (3 days, n=5 each)		
	Measured concentration (mean±SD, μg/l)	RSD (%)	Accuracy (%)	Measured concentration (mean±SD, μg/l)	RSD (%)	Accuracy (%)
1	1.1±0.1	5.1	8.2	1.1±0.1	8.0	5.4
25	24.9±1.7	6.8	−0.4	24.4±2.3	9.5	−2.6
500	529±14	2.6	5.9	517±42	8.3	3.4

rofecoxib in human plasma are summarised in Table 1. The recoveries at concentrations of rofecoxib of 1, 25 and 500 μg/l were 95.6±2.9, 96.1±5.3 and 96.3±1.1%, respectively. The LOQ, according to Shah et al. (RSD, accuracy: <20%) [5] was 1 μg/l (Fig. 2, Table 1).

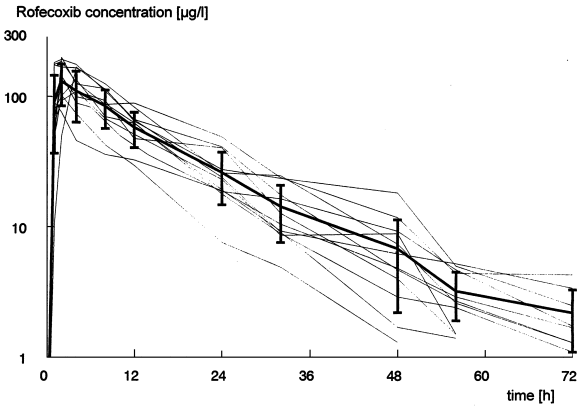

Fig. 5. Plasma concentration–time profiles of rofecoxib (semilogarithmic plot) following oral administration of 12.5 mg monitored in 12 healthy human volunteers.

The validated method has been used successfully to study the pharmacokinetic profile of rofecoxib after minimal oral dose of a single dose (12.5 mg). For these measurements, the calibration curves were established daily using eight plasma concentrations (0, 1, 5, 10, 25, 50, 100, 250 and 500 μg/l). Furthermore, 10% of the measured probes were quality control (QC) samples. QC samples were incorporated in duplicate in at least three different concentrations (1, 25 and 100 μg/l) into each run. The results of the QC samples provided the basis for accepting or rejecting the series.

Fig. 5 presents the plasma concentration–time profiles of rofecoxib in 12 human volunteers following oral administration of 12.5 mg. The pharmacokinetic data are summarised in Table 2.

4. Discussion

The new HPLC–MS^2-based method represents a suitable approach to assess rofecoxib concentrations in human plasma and should be easily extendable to

Table 2
Pharmacokinetic data after oral administration of rofecoxib (12.5 mg) to 12 human volunteers

Volunteer	Gender	Age (years)	Mass (kg)	t_{max} (h)	c_{max} (μg/l)	AUC_∞ (μg h/l)	$t_{1/2}$ (h)
1	Male	35	93	2	145	1628	13.5
2	Male	23	75	2	199	2606	13.3
3	Male	27	86	4	109	1980	9.5
4	Female	27	65	4	168	2321	9.8
5	Male	29	78	2	103	1016	9.7
6	Male	27	84	2	128	1739	10.0
7	Female	25	76	1	91	1204	13.6
8	Female	27	63	2	185	2995	12.6
9	Male	28	80	2	169	2806	9.7
10	Male	27	89	4	135	1954	14.2
11	Male	28	75	2	192	2364	7.3
12	Female	28	82	2	143	1844	12.1

other matrices. In contrast to the HPLC assay [3], no derivatisation procedures are necessary and the assay is very rapid.

In contrast to Chavez-Eng et al. who used a API III Plus triple quadrupole spectrometer (PE-Sciex), we used an Finnigan Mat LCQ ion trap spectrometer for the determination of rofecoxib. Mass analysis was performed in the positive ion mode. With this instrumentation, a main daughter different from Chavez-Eng et al. was detected without endogenous interferences (315→297 vs. 315→192 [4]). This method turned out to be more sensitive (1 μg/l) than Chavez-Eng et al. described for the positive ion mode (10 μg/l). This limit is required to meet the practical needs for profiling therapeutic concentrations in humans. After oral administration of the lowest rofecoxib single dose recommended for therapy, in each volunteer more than 95% of the AUC_{∞} were confined by concentrations above the LOQ.

In the positive ion mode the products resulting from the selected fragmentation reaction of rofecoxib and celecoxib yielded approximately 70% and 50% of all daughters registered, and could be explained by losses of H_2O and HF, respectively. The quantification assay for rofecoxib has been validated in the concentration range from 1 to 500 μg/ml demonstrating a highly reliable precision and accuracy; no signal interferences from endogenous compounds have been observed. Further quality controls revealed adequate analyte stability under all conditions applied.

For quantification of rofecoxib the sample run time was shortened to 4 min vs. 7.5 min [4].

The negative ion method described by Chavez-Eng et al. was tested using different mobile phases containing also variable amounts of formic acid and ammonium acetate. The same daughters were observed, but the performance was not sufficient. Therefore the negative ion technique was not satisfactory with the LCQ instrumentation.

Despite the fact that rofecoxib is one of the most successful new drugs world-wide, individual pharmacokinetic data are rare. Only one paper published in 2000 by Depre et al. [7] discusses individual pharmacokinetic data after higher doses. So, the validated method was used to study the pharmacokinetic profile of rofecoxib in 12 healthy volunteers after administration of the lowest daily dose recommended (12.5 mg).

The pharmacokinetic data (Table 2) emerged with relatively low interindividual variability (t_{max}: 2.4±1.0 h, c_{max}: 147±34 μg/l, AUC_{∞}: 2038±581 μg h/l and $t_{1/2}$: 11.3±2.1 h) and the characteristics are in accordance with the parameters observed by Depre et al. (1×25 mg; c_{max}: 203±52 μg/l, AUC_{∞}: 2941±659 μg h/l and $t_{1/2}$: 9.0±2.7 h [7]). The data given in the product information Vioxx after application of 25 mg are t_{max}: 2–4 h, c_{max}: 305 μg/l (steady state), AUC_{∞}: 3870 μg h/l and $t_{1/2}$: 17 h (accumulation half life) [8]. On average, our calculated half life is shorter than the half life from the published product information.

In conclusion, this new analytical approach features a suitable method to assess the pharmacokinetic of the standard rofecoxib regimen in humans.

Acknowledgements

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