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Determination of Rofecoxib (MK-0966), a cyclooxygenase-2 inhibitor, in human plasma by high-performance liquid chromatography with tandem mass spectrometric detection

C.M. Chavez-Eng*, M.L. Constanzer, B.K. Matuszewski

Merck Research Laboratories, West Point, PA 19486, USA

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Abstract

A method for the determination of 4-(4-methanesulfonylphenyl)-3-phenyl-5H-furan-2-one (Rofecoxib, Vioxx, MK-0966, **I**) a cyclooxygenase-2 inhibitor, in human plasma has been developed. The method is based on high-performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization tandem mass spectrometric (APCI-MS–MS) detection in negative ionization mode using a heated nebulizer interface. Drug and internal standard (**II**) were isolated from basified plasma using liquid–liquid extraction. The organic extracts were dried, reconstituted in mobile phase and injected into the HPLC-MS–MS system. Compounds **I** and **II** were chromatographed on a narrow bore (100 mm×3.0 mm) C_{18} analytical column, with mobile phase consisting of acetonitrile:water (1:1, v/v) at a flow-rate of 0.4 ml/min. The MS–MS detection was performed on a PE-Sciex API III Plus tandem mass spectrometer operated in selected reaction monitoring mode. The parent→product ion combinations of m/z 313→257 and 327→271 were used to quantify **I** and **II**, respectively, after chromatographic separation of the analytes. The assay was validated in the concentration range of 0.1 to 100 ng/ml of plasma. The precision of the assay (expressed as coefficient of variation) was less than 10% at all concentrations within the standard curve range, with adequate assay accuracy. The effect of HPLC mobile phase components on the ionization efficiency and sensitivity of detection in the positive and negative ionization modes, and the detailed description of all necessary steps involved in the assay for **I** in plasma are presented.

Keywords: Rofecoxib; Cyclooxygenase-2 inhibitor

1. Introduction

Reduction of prostaglandin synthesis by direct inhibition of cyclooxygenase (COX, prostaglandin-endoperoxide synthase) is believed to be the mechanism of nonsteroidal antiinflammatory drugs [1]. There are two forms of COX, namely, COX-1 and COX-2. COX-1 is expressed in tissues and is involved in the physiological functions of prostaglandins, while COX-2 is inducible and is expressed in inflammatory cells and in kidney [2–4]. Hence, specific inhibitors of COX-2 are potential antiinflammatory agents which will not produce the toxicity associated with the inhibition of COX-1 derived prostaglandins. Compound **I** (4-(4-methanesulfonylphenyl)-3-phenyl-5H-furan-2-one, MK-0966) has been developed as a specific COX-2 inhibitor for treating acute pain and chronic inflammatory disorders without gastric side effects associated with the use of COX-1 inhibitors.

*Corresponding author.

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Concentrations of **I** in biological fluids from the majority of clinical pharmacokinetic studies with **I** were determined using highly sensitive high-performance liquid chromatographic (HPLC) methods based on postcolumn photochemical conversion of **I** to a substituted phenanthrene analog followed by fluorescence detection [5]. The selectivity, sensitivity and speed of analysis of this HPLC-fluorescence (FLU) method were comparable or even better than many typical HPLC-MS–MS assays. However, it was of interest to develop a HPLC-MS–MS method for **I** to serve as a back-up method for analyses of human plasma samples in cases when confirmatory data were needed. In addition, the availability of the HPLC-MS–MS method for unlabeled **I** could form a basis for the future development of simultaneous assays for determination of unlabeled and stable isotope labeled analogs of **I** that may be utilized in a number of mechanistic and bioavailability studies with **I**. Atmospheric pressure chemical ionization (APCI) HPLC-MS and HPLC-MS–MS techniques have proven to be of great use for both the identification and quantification of drugs and metabolites in biological fluids at very low concentrations. Some recent representative examples from our laboratories are listed in Refs. [6–11]. In APCI, ionization occurs by proton/or electron transfer reactions and is mediated by the gas phase acidities/or basicities of the reagent ions and the compounds of interest. The effect of mobile phase components on sensitivity in APCI HPLC-MS has been recognized [12] and extensively investigated by Schaefer and Dixon [13]. Decrease in ionization efficiencies of **I** and internal standard (**II**) due to the presence of modifiers in the mobile phase was observed during the HPLC-MS–MS method development for these compounds. The proper choice of mobile phase and the type of ionization mode (positive or negative ionization modes) were found to be critical to the successful development of a sensitive assay for **I** and will be discussed in detail in this paper.

2. Experimental

2.1. Materials and reagents

Rofecoxib (MK-0966, Vioxx, **I**) and internal standard (I.S., **II**) were synthesized at Merck (Rahway, NJ, USA). Ammonium acetate was purchased from Sigma (St. Louis, MO, USA). Formic acid was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Fisher (Fair Lawn, NJ, USA). Control human plasma was obtained from Biological Specialties (Lansdale, PA, USA)

2.2. Instrumentation

The HPLC system consisted of a Perkin Elmer Biocompatible Binary pump 250, a WISP 715 Autoinjector (Waters-Millipore, Milford, MA, USA) and an API III Plus triple quadrupole tandem mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer interface. In the exploratory part of the study, ion-spray (ISP) interface was also utilized.

2.3. Chromatographic conditions

The mobile phase consisted of 50% acetonitrile and 50% of water and was pumped at a flow-rate of 0.4 ml/min. Chromatography was performed on a YMC ODS AQ 20×3 mm guard column coupled to YMC ODS AQ 100×3 mm, 3 μm analytical column. The total runtime was 7.5 min with **I** eluting at 5.0 min and I.S. at 6.5 min after injection. A number of other mobile phases were initially evaluated including mixtures of acetonitrile with water containing 0.1% formic acid or 0.1% formic acid in 10 m*M* ammonium acetate.

2.4. Mass spectrometric conditions

The mass spectrometer state file parameters were initially optimized by infusion of analyte solutions using ISP interface in the presence of buffers (0.1% formic acid, 10 m*M* ammonium acetate). For actual analyses, the mass spectrometer was interfaced to the HPLC system via a heated nebulizer. Nebulizer (air) pressure was set at 80 p.s.i., and curtain gas (N_2) flow at 0.9 l/min. Positive/or negative chemical ionization was effected by the corona discharge needle (±4 μA) and the sampling orifice potential was set at ±40 V. For positive ionization mode, the first quadrupole, Q1 was set to monitor the protonated molecules $(M+H)^+$ at m/z 315 for drug and

m/z 329 for I.S. with collision-induced fragmentation at Q2 (collision gas argon, $275 \cdot 10^{13}$ atoms/cm^2), and monitoring the product ions via Q3 at m/z 192 for **I** and m/z 311 for I.S.. In negative ionization mode, the first quadrupole, Q1 was set to monitor the deprotonated molecules $(M-H)^-$ at m/z 313 for drug and m/z 327 for I.S. with collision-induced fragmentation at Q2 (collision gas argon, $275 \cdot 10^{13}$ atoms/cm^2), and monitoring the product ions via Q3 at m/z 285 and 257 for **I** and m/z 271 for I.S. The electron multiplier setting was ± 3.0 kV and detector electronics were set to counts of 10. Dwell time was 400 ms.

2.5. Data acquisition and analysis

Data acquisition and analyses were performed using RAD and MacQuan software (PE-Sciex). Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted $(1/y^2)$ linear least square regression of the calibration line constructed from the peak area ratios of drug to I.S. vs. drug concentration.

2.6. Standard preparation

A standard stock solution of **I** (1.0 mg/ml) was prepared in acetonitrile. Subsequent dilutions were made in acetonitrile to give the following concentrations: 0.001, 0.0025, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 μg/ml. A standard stock solution of I.S. was prepared as 1.0 mg/ml in acetonitrile and subsequent dilutions were made to prepare 10 μg/ml working I.S. standard solution.

The plasma standard line was determined by spiking 1 ml of control human plasma with 100 μl each of the working standard of **I** and I.S. solutions and extracted as described in Sample preparation.

Quality control (QC) plasma samples at 0.75 and 75 ng/ml were prepared by diluting 1.0 ml of 0.075 μg/ml and 1.0 ml of 7.5 μg/ml solution (from a new weighing) to a total volume of 100 ml control human plasma, respectively. Aliquots (1.25 ml) of these solutions were transferred to 2-ml plastic tubes, stored at -20°C, and were analyzed to determine the freeze–thaw and long-term stability of **I** during storage.

2.7. Sample preparation

Initially, during validation of the method in positive ionization mode, the extraction procedure from plasma as developed earlier [5] was adopted. Briefly, 1 ml of plasma was extracted with 8 ml of the mixture of hexane–methylene chloride (1:1, v/v); organic extracts were evaporated to dryness and the residues were reconstituted in 1 ml of acetonitrile followed by 1 ml of water. One hundred and fifty μl of this solution was injected into the HPLC-MS–MS system. The separation was performed on a Keystone BDS C_{18} (100×4.6 mm, 5 μm) analytical column protected with a BDS C_{18} (20×4.6 mm, 5 μm) guard column. The mobile phase flow-rate was 1.0 ml/min. Under these conditions, the retention times of **I** and **II** were 3.0 and 3.6 min, respectively.

The extraction method was later modified during validation of the assay in the negative MS–MS mode. It was found that much cleaner extracts were obtained when methyl-*tert*.-butyl ether (MTBE) was utilized as an extraction solvent and plasma was basified before extraction. The final procedure in the negative ionization mode was as follows: To 1 ml of plasma, 100 μl each of the working standard and I.S. solutions, followed by 1 ml of pH 9.8 carbonate buffer were added. Eight ml of MTBE was transferred to the tube and the mixture was rotated-mix for 15 min. The organic layer was separated, transferred to a clean tube, and after evaporation to dryness, the residue was reconstituted in 50 μl acetonitrile. After vortexing for one min, 50 μl of water was added and the mixture was vortexed and sonicated for 15 min. Twenty-five μl of this solution was injected into the HPLC-MS–MS system.

3. Results and discussion

3.1. Effect of mobile phase modifiers on the ionization efficiency and sensitivity of detection

The Sciex mass spectrometer provides two ways for interfacing HPLC with MS: heated pneumatic nebulization and ISP or turbo ISP (TISP). Although ISP or TISP are widely used in quantitative analyses, the technique has some limitations, namely, a limited dynamic range [14,15] and a higher probability of exhibiting a matrix effect [15–18]. APCI using

heated pneumatic nebulization has been widely used in quantitative analysis and is becoming even more popular because of its sensitivity, ease of operation, and less likelihood of ion suppression or enhancement. This heated nebulizer ionization technique is especially useful for analysis of thermally stable analytes and allows direct interfacing of the MS system with HPLC performed using large diameter (4.6 mm) analytical columns. Thus, the heated nebulizer was chosen as an interface during development of the assay for **I**.

Initially, ionization of **I** was studied using both ISP and heated nebulizer interfaces in standard solutions of **I** containing typical modifiers and/or buffers (0.1% formic acid and 10 m*M* ammonium acetate). When **I** was infused using the ISP interface under positive ionization mode, formation of ions was observed. However, practically no ionization was observed when mobile phases containing modifiers were utilized using the heated nebulizer interface. In order to study the effect of solvent (mobile phases) on the efficiency of ionization of **I** using the heated nebulizer interface, a series of experiments in different solvents was performed. Using the optimized MS state file parameters established under ISP conditions, the orifice potential was reoptimized after switching from ISP to the heated nebulizer interface. This was done by flow injection analysis of 10 μg/ml solutions of **I** in three different mobile phases. The mobile phases consisted of 80% acetonitrile and 20% of neat water or water containing modifiers. Modifiers were added to the aqueous portion of the mobile phase as either 0.1% formic acid or as a mixture of 0.1% formic acid and 10 m*M* ammonium acetate solutions.

Full scan (200 to 1000 mass range) mass spectra (Q1 spectra) were acquired and compared between the three mobile phases. These mass spectra indicated the presence of intense $(M+H)^+$ ions of **I** at *m/z* 315 when the mobile phase did not contain any modifiers, and practically none or very little of $(M+H)^+$ ions were present when the mobile phase contained an acid/or an acid and buffer mixture. The product ion mass spectrum of the $(M+H)^+$ ions of **I** (Fig. 1) showed intense fragments at *m/z* 297 [$(M+H)^+-H_2O$], *m/z* 269 [$(M+H)^+-(H_2O+CO)$], and *m/z* 192 [$(M+H)^+-(H_2O+CO+Ph)$]. The precursor→product ion combinations at *m/z* 315→297 and 315→269 were used in multiple reaction monitoring (MRM) mode to compare intensities when the same amounts of **I** were injected using mobile phases with and without modifiers. Mean peak areas for **I** obtained using acetonitrile–water as a mobile phase were more than 30-times higher than the mean peak areas of **I** obtained when mobile phases containing modifiers were utilized (Table 1). These MS data indicated that compound **I,** a neutral molecule not containing easily ionizable groups, had probably lower proton affinity than ions generated when modifiers were present in the mobile phase.

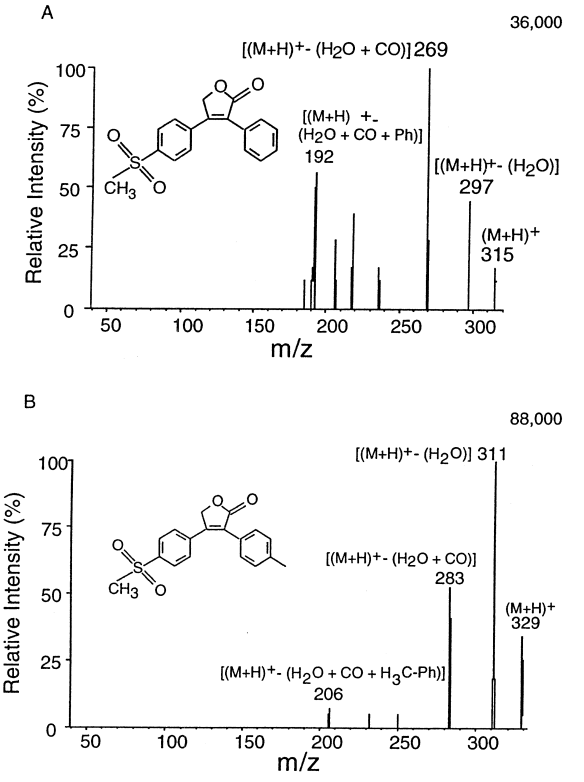

Fig. 1. Chemical structures and positive product ion mass spectra of the protonated molecules of **I** (**A**) (*m/z* 315) and internal standard, **II** (**B**) (*m/z* 329).

Based on these findings, an HPLC-MS–MS method with heated nebulization in positive ionization mode utilizing acetonitrile–water as the mobile phase was initially developed.

Table 1
Peak areas of **I**[a] obtained after flow injection analysis in different mobile phases: (1) acetonitrile–water (80:20, v/v); (2) acetonitrile–water containing 0.1% formic acid and 10 m*M* ammonium acetate (80:20, v/v), under optimized state file conditions

	Mobile phase 1		Mobile phase 2	
Injection	m/z 315→297	m/z 315→269	m/z 315→297	m/z 315→269
1	619 331	858 680	2377	23 122
2	668 687	781 114	2197	26 855
3	662 286	646 995	2263	23 050
4	546 288	716 093	2016	24 363
Mean	624 148	750 721	2213	24 348
SD	56 343	90 438	1509	1777
C.V. (%)	9.0	12.0	6.8	7.3

[a] Integration parameters were adjusted for baseline noise to be similar in mobile phases 1 and 2.

3.2. HPLC-MS–MS assay using heated nebulizer in positive ionization mode

The product ion mass spectra of $(M+H)^+$ ions of **I** at m/z 315 and **II** at m/z 329 are shown in Fig. 1. Although the sensitivity of detection using the precursor→product ion combination at m/z 315→269 for **I** was better than at m/z 315→192, the latter was used due to the presence of endogenous interferences when the former channel was utilized for detection. The precursor→product ion combination at m/z 329→311 in MRM mode was used to monitor the internal standard.

Sample preparation was similar as in the HPLC/FLU method, as described in Section 2.7. Results of the initial validation (Table 2) showed poor sensitivity (limit of quantification ≥10 ng/ml) and relatively poor precision (expressed as coefficient of variation, C.V.) of 11.9 and 10.5% at 10 and 20 ng/ml, respectively. Clearly, a more sensitive and reliable method was needed to support studies with low doses of **I**.

3.3. HPLC-MS–MS detection in negative ionization mode

In order to determine **I** at concentrations at or below 1 ng/ml and to support a number of mechanistic and bioavailability studies with stable isotope labeled **I**, a method with high sensitivity and precision was required. Since the efficiency of ionization under positive ionization conditions was poor, the ionization efficiency under negative ionization conditions was evaluated and the effect of the mobile phase components on the ionization efficiency and sensitivity of detection was investigated. Similarly,

Table 2
Precision and accuracy of the assay of **I** in human plasma under positive APCI-MS–MS ionization conditions

Nominal conc. (ng/ml)	Calculated conc. (ng/ml)	Precision[a] C.V. (%)	Accuracy[b] (%)
10.0	10.6	11.9	106.0
20.0	19.3	10.5	96.5
50.0	51.8	4.2	103.6
100.0	95.6	7.4	95.6
200.0	198.2	5.3	99.1
500.0	517.8	7.7	103.6
1000.0	1057.2	4.4	105.7

[a] Expressed as coefficient of variation (C.V., %); $n=5$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)]×100.

as in the positive ionization mode, the best sensitivity of detection was obtained using a mixture of acetonitrile–water as the mobile phase *without* the presence of any modifiers. The HPLC and MS conditions were optimized in the negative mode and under these optimized conditions, the mass spectrum (Q1 scan) indicated the presence of intense $(M-H)^-$ ions of **I** and **II** at m/z 313 and 327, respectively. The product ion mass spectra of these deprotonated molecules of **I** and **II** are presented in Fig. 2.

Comparison of the sensitivity of detection for **I** under positive and negative ionization conditions was performed by injecting solutions containing low to high concentrations of **I** and a constant amount of **II**. The overall sensitivity of detection of **I** in negative ionization mode was about 25-times greater than in the positive ionization mode. As shown in Fig. 3(A‴, B‴), injection of 0.2 ng of **I** and 7.5 ng of **II** on the column resulted in signal-to-noise ratios (S/N) of 47 and 875 for **I** and **II**, respectively under negative ionization mode. Injection of the same solution as described, under positive ionization mode (Fig. 3, A′, B′) showed no integrable peak of **I** and a S/N ratio of 295 for **II**. S/N ratios were calculated directly by MacQuan software.

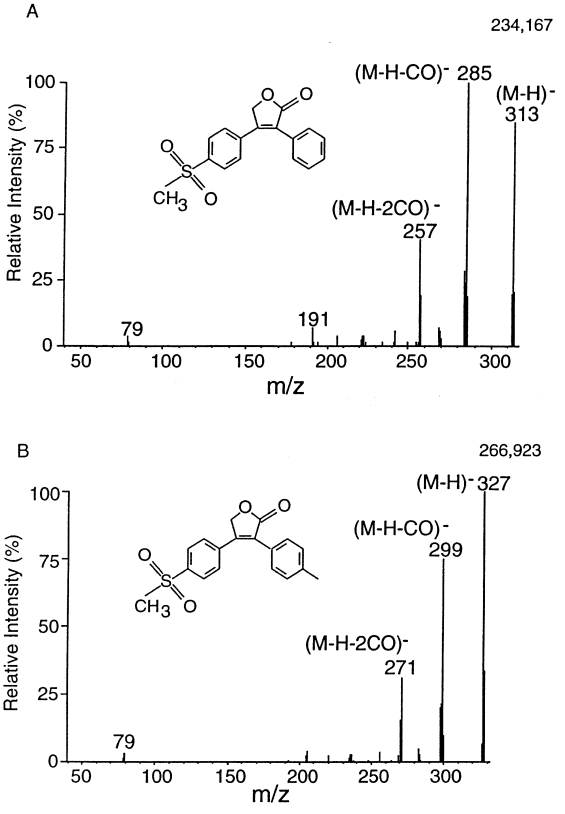

Fig. 2. Chemical structures and negative product ion mass spectra of the deprotonated molecules of **I** (**A**) (m/z 313) and internal standard, **II** (**B**) (m/z 327).

3.4. HPLC-MS–MS method validation under negative ionization conditions

Assay validation was performed using sample preparation as described in Section 2.7, and chromatographic and mass spectrometric conditions as described in Sections 2.3 and 2.4, respectively. The precursor→product ion combinations of m/z 313→257 and 327→271 were utilized for quantification of **I** and **II**, respectively, after chromatographic separation of the analytes. Although the sensitivity of detection for **I** using MRM at m/z 313→285 was higher than at 313→257, the latter was used for quantification due to the presence of endogenous impurities when the former channel was utilized. For the same reason, the internal standard **II** was monitored at m/z 327→271 instead of 327→299. The intraday assay precision and accuracy were assessed in control human plasma in the concentration range of 0.1 to 100.0 ng/ml. The precision of the assay was less than 10% at all concentrations within the standard curve range with adequate assay accuracy as shown in Table 3.

The selectivity of the method was assessed by analyzing extracts from five lots of plasma from different sources. **I** and I.S. were chromatographically separated from each other and endogenous interferences. The representative chromatograms of human plasma analyzed under negative ionization conditions are presented in Fig. 4. The absence of a ''cross-talk'' effect from I.S. to parent drug is depicted in chromatograms A″ and B″ where I.S. at the concentration used in the assay (10 ng/ml) was injected and no response at the MS–MS channel used for monitoring **I** was observed.

Analyses of QC samples at low concentration (0.75 ng/ml) gave a mean concentration of 0.72 ±0.045 ng/ml ($n=5$). After the first and second freeze–thaw of the QC sample, the assayed concentrations were 0.80 and 0.77 ng/ml, respectively.

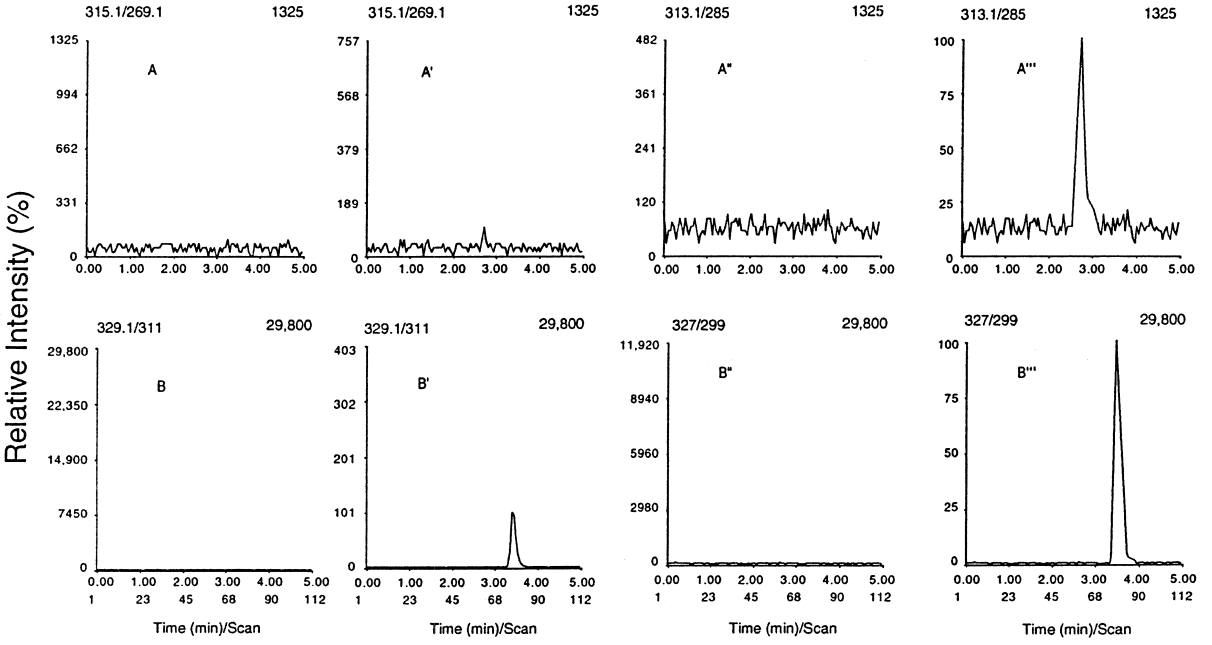

Fig. 3. Representative HPLC-MS–MS chromatograms of the neat standards obtained in MRM in positive ionization mode at m/z 315→269 for **I**, (Chromatograms A, A′) and m/z 329→311 for **II**, (Chromatograms B, B′) and in the negative ionization mode at m/z 313→285 for **I**, (Chromatograms A″ A‴) and at m/z 327→299 for **II**, (Chromatograms B″, B‴); chromatograms A and B — neat solvent; chromatograms A′ and B′ — neat standard of **I** and **II** (0.2 and 7.5 ng on column, respectively) in positive ionization mode; A″ and B″ — neat solvent; chromatograms A‴ and B‴ — neat standard of **I** and **II** (0.2 and 7.5 ng on column, respectively) in negative ionization mode. The numbers in upper right hand corner of chromatograms correspond to the peak heights expressed in arbitrary units.

Similarly, analyses of QC samples at high concentration (75 ng/ml) gave a mean concentration of 75.7 ±1.9 ng/ml ($n=5$). After the first and second freeze–thaw of the high QC sample, the assayed concentrations were 76.5 and 77.7 ng/ml, respectively, confirming the good stability of **I** and good precision and accuracy of the assay.

Table 3
Precision and accuracy of the assay of **I** in human plasma using negative APCI-MS–MS

Nominal conc. (ng/ml)	Calculated conc. (ng/ml)	Precision[a] C.V. (%)	Accuracy[b] (%)
0.10	0.10	6.7	100.0
0.25	0.27	6.8	108.0
0.50	0.51	9.1	102.0
1.00	1.02	5.0	102.0
5.00	4.98	1.8	99.6
10.00	9.96	2.5	99.6
50.00	49.53	1.9	99.1
100.00	98.03	4.0	98.0

[a] Expressed as coefficient of variation (C.V., %); $n=5$.
[b] Expressed as [(mean calculated concentration)/(spiked concentration)]×100.

3.5. Analyses of clinical samples

The HPLC-MS–MS assay in the negative ionization mode was used to support a clinical study in human subjects. Representative concentrations of **I** in plasma from four subjects after a 25-mg oral dose of **I** are presented in Fig. 5.

The development and validation of an assay for the simultaneous determination of unlabeled and a stable isotope labeled analog of **I** used in bioavailability study will be reported separately.

In conclusion, a sensitive and selective HPLC-MS–MS method has been developed for the determination of Rofecoxib in human plasma in the concentration range of 0.1–100 ng/ml. The successful development of this assay required an extensive evaluation of the ionization efficiencies of this

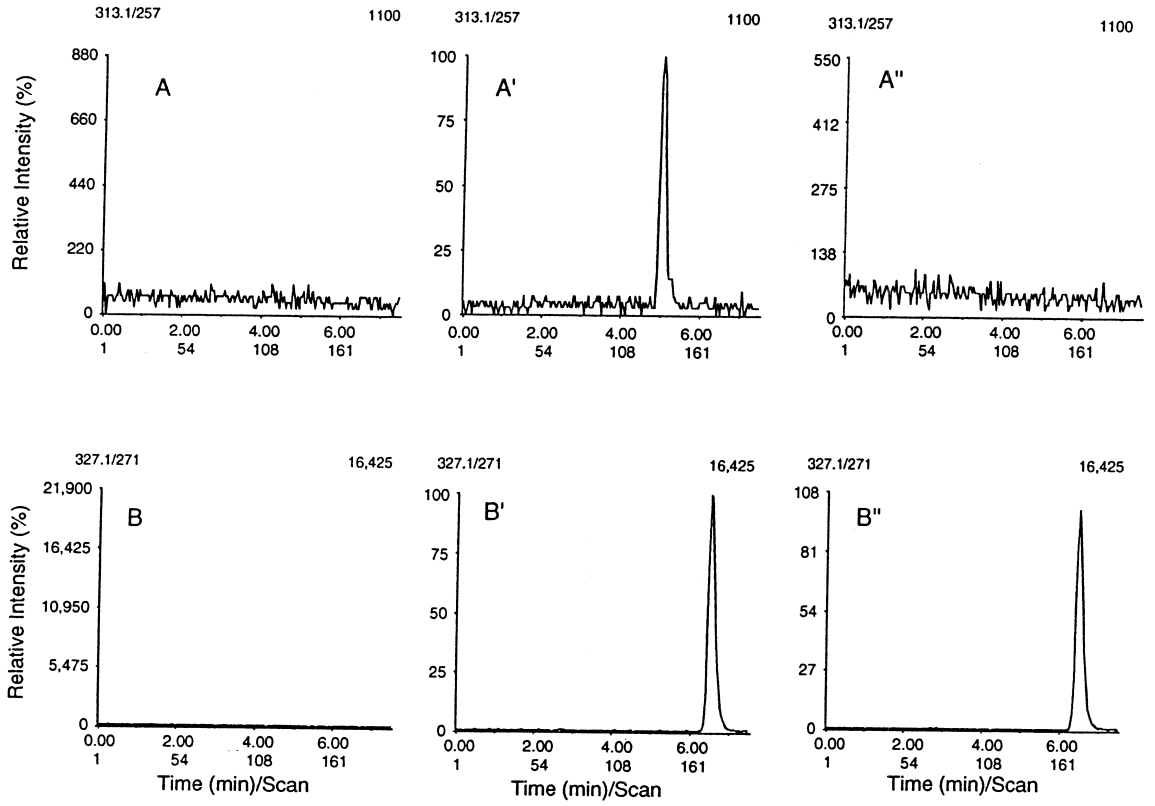

Fig. 4. Representative HPLC-MS–MS chromatograms of the plasma extracts obtained by multiple reaction monitoring at m/z 313→257 for **I**, (Chromatograms A, A′, A″) and m/z 327→271 for **II**, (Chromatograms B, B′, B″); chromatograms A and B — extracts of control plasma; chromatograms A′ and B′ — extracts of control plasma spiked with 0.5 ng/ml of **I** and 10 ng/ml of **II**; chromatograms A″ and B″ — extracts of control plasma spiked with 10 ng/ml of **II**. The numbers in upper right hand corner of chromatograms correspond to the peak heights expressed in arbitrary units.

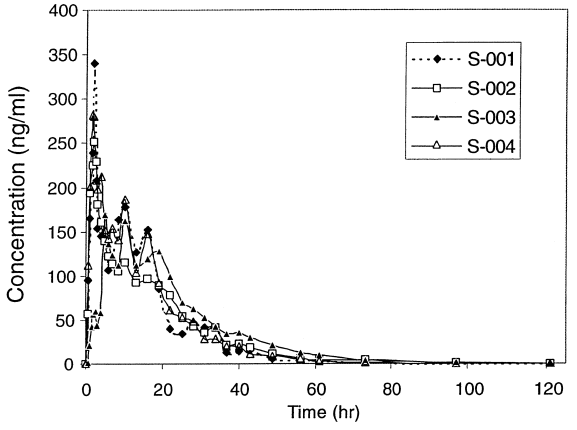

Fig. 5. Representative concentrations of **I** in plasma from four subjects receiving a 25 mg oral dose of **I**.

relatively difficult to ionize molecule in different solvents (mobile phases), and utilization of negative rather than positive ionization conditions for MS–MS detection.

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