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High-performance liquid chromatographic–tandem mass spectrometric evaluation and determination of stable isotope labeled analogs of rofecoxib in human plasma samples from oral bioavailability studies

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Abstract

A method for the simultaneous determination of a cyclooxygenase-2 inhibitor, 4-(4-methanesulfonylphenyl)-3-phenyl-5H-furan-2-one (rofecoxib, **I**) and [$^{13}C_7$]rofecoxib, (**II**), in human plasma has been developed to support the clinical oral bioavailability (BA) study of **I**. The method is based on high-performance liquid chromatography (HPLC) with atmospheric pressure chemical ionization tandem mass spectrometric (APCI-MS–MS) detection in the negative ionization mode using a heated nebulizer interface. Two different stable isotope labeled analogs of **I** were initially evaluated for their use as intravenous (i.v.) markers in the BA study. [$^{13}CD_3$]Rofecoxib was shown to be isotopically unstable in plasma and water containing solvent and an efficient deuterium exchange prevented its use in the study. On the other hand, the isotopic integrity of the subsequently synthesized [$^{13}C_7$]rofecoxib (**II**) was maintained, as expected, in plasma and other solvent systems. The results of these experiments clearly demonstrated the need for the careful evaluation of the isotopic integrity of the stable isotope labeled compound for the successful utilization of these compounds in BA studies and also as internal standards in the quantitative analysis of drugs in biological fluids. After liquid–liquid extraction of **I**, **II**, and internal standard (**III**) from plasma, the analytes 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 0.5 ml/min. The MS–MS detection was performed on a PE Sciex API III Plus tandem mass spectrometer operated in the selected reaction monitoring mode. The precursor→product ion combinations of m/z 313→257, 320→292, and 327→271 were used to quantify **I**, **II**, and **III**, respectively. The assay was validated in the concentration range of 0.1 to 100 ng/ml of plasma for both **I** and **II**. The precision of the assay (expressed as relative standard deviation) was less than 10% at all concentrations within the standard curve range, with adequate assay accuracy. The assay was utilized to support the clinical BA study in which oral doses of **I** were administered together with an i.v. dose of **II** to determine the oral BA of rofecoxib at 12.5- and 25-mg doses.

Keywords: Bioavailability study; Rofecoxib

1. Introduction

Compound **I** [4-(4-methanesulfonylphenyl)-3-phenyl-5H-furan-2-one, rofecoxib] has been de-

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veloped at the Merck Research Labs. and was recently approved by the US Food and Drug Administration (FDA) as a specific COX-2 inhibitor for treating acute pain and chronic inflammatory disorders. It is being marketed in the US under the trade name VIOXX.

The use of stable isotope labeled compounds to study the pharmacokinetics of drugs has been reviewed in depth in two review papers [1,2]. The labeled compounds have been employed to study absorption, distribution, metabolism, and excretion of drugs in animal and human subjects. Most commonly, stable isotope labeled analogs of drugs are utilized to determine absolute or relative bioavailability (BA) of a test compound [3–11]. In these BA studies, the unlabeled and labeled drugs are administered simultaneously by a different route of administration (for example oral and intravenous, i.v.), and plasma concentrations vs. time profiles are used to calculate the necessary pharmacokinetic parameters to determine the oral BA. To conduct these studies, validated quantitative methods are required for the simultaneous determination of the labeled and an unlabeled drug.

Mass spectrometry, in general, has been the most widely used analytical technique for analysis of samples containing stable isotopes. A number of gas chromatographic–mass spectrometric (GC–MS) assays have been developed for simultaneous quantification of labeled and unlabeled drugs in biological fluids [3–5]. GC–MS usually provides high selectivity and sensitivity for the analysis of compounds that are volatile and would not degrade at high temperatures. For non-volatile or thermally labile compounds, high-performance liquid chromatography (HPLC) is often the method of choice. Recently, HPLC with mass spectrometric (HPLC–MS) or tandem mass spectrometric (HPLC–MS–MS) detection became the method of choice for the quantitative determination of drugs in biological fluids. Several of these HPLC–MS–MS or HPLC–MS assays were utilized for the simultaneous determination of labeled and unlabeled drugs in serum or plasma [6–10].

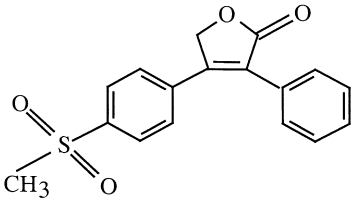
The major concern in the utilization of stable isotope labeled compounds in the preclinical and clinical studies is the issue of isotopic integrity of the labeled compound in the environment (solvent or a biofluid) in which the compound is studied. Lack of the isotopic integrity of the label due, for example, to deuterium or oxygen-18 exchange, may prevent the inappropriately labeled molecule being used in the study. Therefore, isotopic integrity of the labeled compound needs to be carefully evaluated before it is utilized in drug metabolism studies. In the case of **I**, an isotopically labeled [$^{13}CD_3$]rofecoxib was initially synthesized but it was demonstrated that due to an efficient deuterium exchange in biofluids and other water-containing solvents it could not be utilized in the preclinical and clinical studies. The results of these isotopic integrity studies necessitated the subsequent synthesis of [$^{13}C_7$]rofecoxib (**II**). The full integrity of carbon-13 labels in this molecule was demonstrated, as expected. The results of the isotopic integrity studies in biofluids and in other solvents for **I** and **II** are provided.

In order to use **II** as an i.v. marker for the human BA study, a HPLC–MS–MS method for the simultaneous determination of unlabeled (**I**) and stable isotope labeled (**II**) was required and was developed. The methodology for the determination of unlabeled **I** in human plasma was described by us earlier [12]. In addition to the studies of the isotopic integrity of the labeled compounds and the assessment of the isotopic content of **I** and **II** from MS and MS–MS responses, the development of a simultaneous assay for **I** and **II** required demonstration of the absence of cross-talk effects, a careful assessment of assay selectivity, and the demonstration of the utility of the method for supporting the human BA study. The results of all these studies and the details of a HPLC–MS–MS method for the simultaneous determination of **I** and [$^{13}C_7$]-labeled rofecoxib (**II**) in human plasma in support of oral BA study with **I** is the subject of this publication.

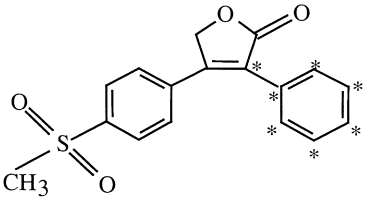
2. Experimental

2.1. Materials and reagents

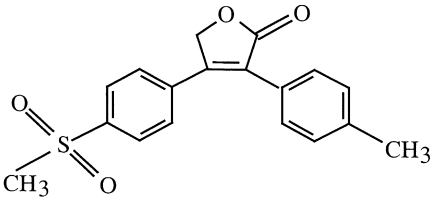
Rofecoxib (VIOXX, **I**) and internal standard (I.S., **III**) (Fig. 1) were synthesized at Merck Research Labs. (Rahway, NJ, USA). [$^{13}C_7$]Rofecoxib (**II**, Fig. 1) and [$^{13}CD_3$]rofecoxib (Fig. 2) were prepared by Dr. M. Braun of the radiosynthesis group and



Rofecoxib, **I**



[$^{13}C_7$] -Rofecoxib, **II**;
Positions of ^{13}C labels are denoted by an asterisk



Internal Standard, **IS**, **III**

Fig. 1. Chemical structures of rofecoxib (**I**), [$^{13}C_7$]rofecoxib (**II**), and internal standard (I.S., **III**).

initially analyzed by GC–MS by Dr. A. Jones of the Department of Drug Metabolism, Merck Research Labs.

Acetonitrile (ACN), methyl-*tert*.-butyl ether, sodium carbonate, and sodium hydrogencarbonate were obtained from Fisher Scientific (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 Perkin-Elmer 200 Series autoinjector, and an API III Plus triple quadrupole tandem mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer (HN) interface.

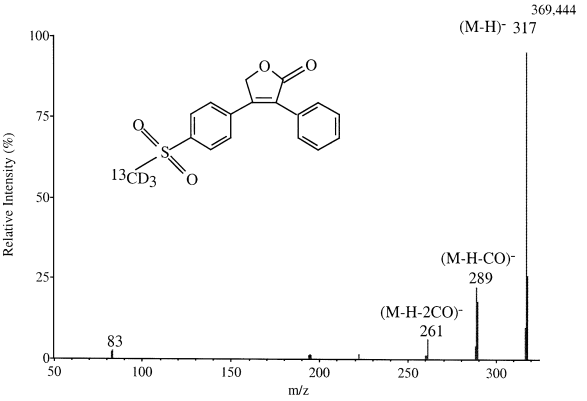


Fig. 2. Chemical structure and negative product ion mass spectrum of the deprotonated molecule of [$^{13}CD_3$]rofecoxib (m/z 317).

2.3. Chromatographic conditions

These conditions were similar to those used in the method for unlabeled **I** [12] with minor changes. The same mobile phase composition was utilized (i.e., acetonitrile–water, 50:50), but the mobile phase flow-rate was increased from 0.4 to 0.5 ml/min. Chromatographic separation was performed on a YMC ODS AQ direct connect guard column (20×2 mm) instead of a threaded guard column (23×4 mm) coupled to a YMC ODS AQ 100×3 mm, 3 μm analytical column. The total runtime was 5.0 min with **I** and **II** eluting at 3.0 min and **III** at 3.9 min after injection.

2.4. Mass spectrometric conditions

The mass spectrometer was interfaced to the HPLC system via a heated nebulizer. Nebulizer (N_2) pressure was set at 80 p.s.i., and curtain gas (N_2) flow at 0.9 l/min (1 p.s.i.=6894.76 Pa). Negative chemical ionization was effected by the corona discharge needle (−4 μA) and the sampling orifice potential was set at −40 V. The first quadrupole, Q1 was set to monitor the deprotonated molecules [M−H]$^-$ at m/z 313, 320, and 327 for **I**, **II**, and **III**, respectively, 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 257, 292, and 271 for **I**, **II**, and **III**, respectively. Q1 and Q3 were operated at unit mass resolution. 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 analyte (**I** or **II**) to I.S. vs. analyte concentration.

2.6. Standard preparation

Standard stock solutions of **I** and **II** (10 μg/ml) were 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 **III** prepared as 1.0 mg/ml in acetonitrile and subsequent dilutions were made to prepare 10 μg/ml working **III** 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**, **II** and **III** solutions and extracted as described in Section 2.7.

Quality control (QC) plasma samples at 0.75 and 75 ng/ml were prepared by diluting 100 μl of 0.75 μg/ml and 75 μl of 100 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 the analytes during storage.

2.7. Sample preparation

Extraction procedure was similar to that described previously [12] with minor modifications. Briefly, 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. A 7-ml volume of methyl-*tert*.-butyl ether was transferred to the tube and the mixture was rotate-mixed for 15 min. The tubes were centrifuged at 3000 rpm (1500 *g*) for 5 min to affect phase separation. The tubes were then placed in a dry ice–acetone bath to freeze the aqueous layer, the organic layer was decanted into a clean tube and, after evaporation to dryness, the residue was reconstituted in 75 μl acetonitrile. After vortexing for 1 min, 75 μl of water was added and the mixture was vortexed and sonicated for 15 min. This solution was transferred to a centrex filter and the tubes were centrifuged at 4000 rpm (2000 *g*) for 5 min. A 40-μl volume of this solution was injected into the HPLC–MS–MS system.

2.8. Determination of unlabeled rofecoxib, [$^{13}CD_3$]rofecoxib, [$^{13}C_7$]rofecoxib, and an internal standard (**III**) from the MS–MS data

2.8.1. Product ion mass spectra

The MS conditions were optimized in the negative mode and under these conditions, the mass spectra (Q1 scans) indicated the presence of intense $[M-H]^-$ ions of [$^{13}CD_3$]rofecoxib, **I**, **II**, and **III** at m/z 317, 313, 320, and 327, respectively. The product ion mass spectrum of deprotonated ions of [$^{13}CD_3$]rofecoxib (Fig. 2) showed intense fragments at m/z 289 and 261 corresponding to the loss of one or two molecules of carbon monoxide (CO), respectively. Similar fragmentation patterns were observed for **I**, **II**, and **III** with intense fragment ions at m/z 285, 257; 292, 264; and 299, 271, respectively (Fig. 3). From these data, the relative concentrations of unlabeled (**I**), [$^{13}CD_3$]-labeled, and [$^{13}C_7$]-labeled rofecoxib (**II**) were determined by selected reaction monitoring using the precursor→product ion combinations of m/z 313→285 or 313→257, 317→289 or 317→261, and 320→292 or 320→264, respectively. Internal standard (**III**) concentrations were determined by selected reaction monitoring using the precursor→product ion combinations of m/z 327→271.

2.8.2. Assessment of the isotopic content of $^{13}CD_3$- and $^{13}C_7$-labeled rofecoxib from MS or MS–MS responses

This determination was based on the relative MS

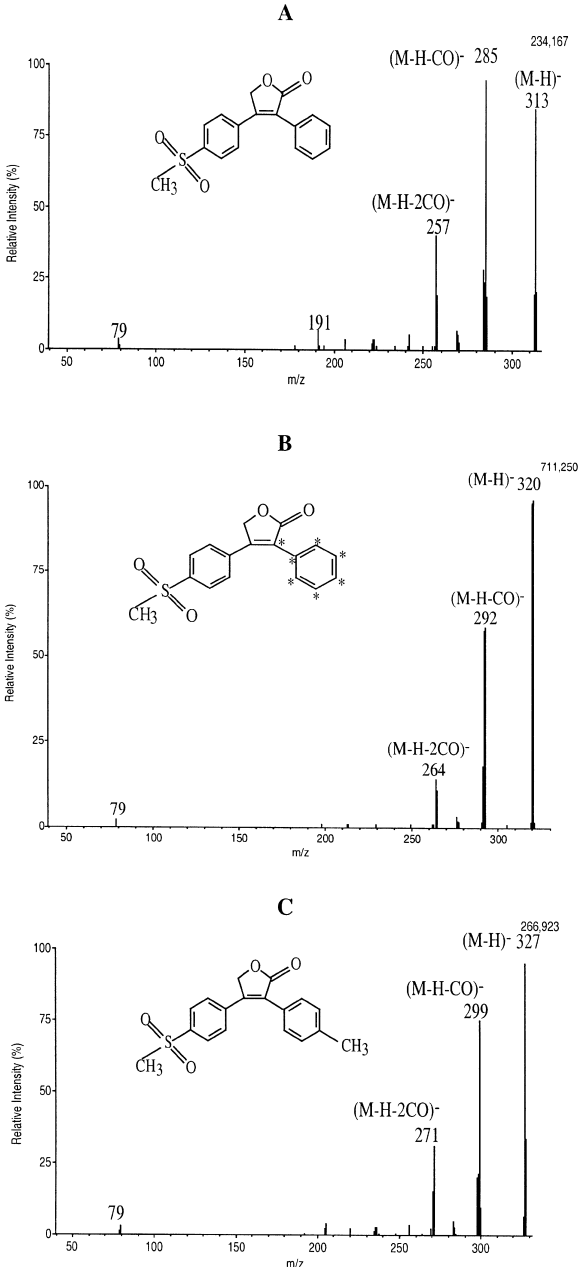


Fig. 3. Chemical structures and negative product ion mass spectra of the deprotonated molecules of **I** (A) (*m*/*z* 313), **II** (B) (*m*/*z* 320), and internal standard, **III** (C) (*m*/*z* 327).

or MS–MS responses in the channels characteristic of unlabeled, $^{13}CD_3$-, and $^{13}C_7$-labeled rofecoxib, after appropriate corrections for natural abundance of all isotopic species and the ''cross-talk'' between MS channels. These calculations were performed in a manner similar to that described by Biemann in the case of electron ionization (EI) single MS spectra [13]. However, the EI-MS response was replaced here by the MS–MS responses from channels corresponding to various isotopic species. For example, the MS–MS responses in the channels characteristic for unlabeled (*m*/*z* 313→285 or 313→257, P), $^{13}CD_3$-labeled (*m*/*z* 317→289 or 317→261, P+4), and $^{13}C_7$-labeled rofecoxib (*m*/*z* 320→292 or 320→264, P+7) using unlabeled standard of rofecoxib were compared with similar responses for the labeled rofecoxib. Although unlabeled rofecoxib gave a major response in the *m*/*z* 313→285 or 313→257 (P) channel, some smaller responses in the channels (P+1), (P+2), (P+3), and (P+4) were also observed. For [$^{13}CD_3$]rofecoxib, major response was observed at (P+4) channel, but there was also smaller response in the channel used for the determination of unlabeled rofecoxib. [$^{13}C_7$]Rofecoxib did not give any responses at the channels characteristic for unlabeled **I**. A number of calculations were needed to subtract the contributions of the unlabeled **I** in all channels of interest from the areas of the $^{13}CD_3$-labeled material observed in the same channels. This was accomplished by calculating the ratios of the responses in channels (P+1), (P+2), (P+3), and (P+4) to the response in channel P of the unlabeled material and by subtracting the contribution of the unlabeled material to channels (P+1), (P+2), (P+3), and (P+4) of the labeled compound. By repeating this process, the real responses in channels characteristic for the labeled material were calculated and the content (%) of the individual labeled species was determined. Calculations were done using Excel program, and a detailed example of calculations and data obtained is shown in Table 1. Similar calculations were performed to assess the content of various isotopic species of [$^{13}C_7$]rofecoxib.

2.8.3. Stability of $^{13}CD_3$- and $^{13}C_7$-labeled rofecoxib

Initial experiments were performed to determine the integrity of the $^{13}CD_3$- and $^{13}C_7$-labeled rofecoxib in different solutions and after addition of [$^{13}CD_3$]-

Table 1
Determination of the isotopic content of [$^{13}CD_3$]rofecoxib and contribution of unlabeled rofecoxib to the response at the [$^{13}CD_3$]rofecoxib channels based on the peak areas of separately injected unlabeled and $^{13}CD_3$-labeled rofecoxib standards at m/z 313→257 (rofecoxib) and m/z 317→261 ([$^{13}CD_3$]rofecoxib)

Standard injected	Peak areas					Ratios			
	313→257 (P)	314→258 (P+1)	315→259 (P+2)	316→260 (P+3)	317→261 (P+4)	(P+1)/P	(P+2)/P	(P+3)/P	(P+4)/P
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
Rofecoxib	3 668 663	607 505	226 481	27 228	4209	0.1656	0.0617	0.0074	0.0011
	3 722 962	594 610	221 424	31 008	3109	0.1597	0.0595	0.0083	0.0008
	3 596 632	647 379	237 169	29 415	5463	0.1800	0.0659	0.0082	0.0015
Mean						0.1684	0.0624	0.0080	0.0011
[$^{13}CD_3$]Rofecoxib	45 695	719 904	294 970	225 788	2 058 807				
	27 683	802 006	290 044	248 442	2 068 664				
	29 420	721 684	301 678	235 512	2 118 510				

	Peak area adjustments									Content (%)				
	Cont. $^{12}CH_3$ to $^{13}CH_3$	$^{13}CH_3$ only	Cont. $^{13}CH_3$ to $^{13}CDH_2$	$^{13}CDH_2$ Only	Cont. $^{13}CDH_2$ to $^{13}CD_2H$	$^{13}CD_2H$ only	Cont. $^{13}CD_2H$ to $^{13}CD_3$	$^{13}CD_3$ Only	Total areas	Rofecoxib	$^{13}CH_3$	$^{13}CDH_2$	$^{13}CD_2H$	$^{13}CD_3$
	J	*K*	*L*	*M*	*N*	*O*	*P*	*Q*	*R*	*S*	*T*	*U*	*V*	*W*
Rofecoxib														
[$^{13}CD_3$]Rofecoxib	7695	712 209	122 787	172 183	73 803	151 985	28 921	2 029 886	3 111 958	1.47	22.89	5.53	4.88	65.23
	4662	797 344	136 000	154 044	75 917	172 525	28 806	2 039 858	3 191 454	0.87	24.98	4.83	5.41	63.92
	4954	716 730	122 533	179 145	75 127	160 385	29 596	2 088 914	3 174 594	0.93	22.58	5.64	5.05	65.80
Mean										1.09	23.48	5.33	5.11	64.98

$J=A0.1684$; $K=B-J$; $L=A0.0624+K0.1684$; $M=C-L$; $N=A0.0080+K0.0624+M0.1684$; $O=D-N$; $P=A0.0011+K0.0080+M0.0624+O0.1684$; $Q=E-P$; $R=A+K+M+O+Q$; $S=A/R\cdot100$; $T=K/R\cdot100$; $U=M/R\cdot100$; $V=O/R\cdot100$; $W=Q/R\cdot100$.

or [$^{13}C_7$]rofecoxib to control human plasma and extraction. Neat standard solutions of labeled compounds were prepared in acetonitrile or acetonitrile–water (50:50, v/v) and were analyzed using the HPLC–MS–MS and/or GC–MS systems. In addition, similar standard solutions of labeled compounds were prepared in cyclooctane and analyzed by GC–MS to determine the initial content of the labeled species in the solid material directly after synthesis. Experiments with human plasma involved preparation of one set of samples that was extracted immediately (0 h, Table 2) after addition of 100 μl of 1.0 or 3.0 μg/ml solution of [$^{13}CD_3$]- or [$^{13}C_7$]rofecoxib to 1 ml of plasma. Second and third sets were prepared in the same manner, and stored at room temperature. Samples from these sets were extracted and analyzed 3 and 6 h (Table 2) after preparation, respectively. The sample preparation and extraction procedures were described in Section 2.7 above, and assessment of isotopic content was performed following procedures described in Section 2.8.2.

2.9. Determination of the ratio of [$^{13}C_7$]rofecoxib to unlabeled rofecoxib in i.v. dosing solutions

The ratio was determined by comparing the HPLC–MS–MS response of a mixture of standards containing **I** and **II** (1:1, w/w) present in the same vehicle as used in i.v. dosing solutions and diluted with mobile phase. The peak area ratios of the response in channel m/z 320→292 for **II** to the response in channel m/z 313→257 for **I** were determined for this standard mixture at four different concentrations. Five replicate injections at each concentrations were made and the mean (n=20) peak area ratio for the 1:1 mixture of standards (PAR_s) was calculated. Similar ratios were determined for the i.v. dosing solutions of all subjects participating in the study. The residual i.v. samples after dosing subjects in the clinic were diluted with mobile phase in the same manner as the standards, and the peak area ratio of the response in channels m/z 320→292 to m/z 313→257 was determined. The dosing solution samples were injected five times

Table 2
Isotopic integrity of the $^{13}CD_3$- and $^{13}C_7$-labeled rofecoxib

Experiment[a]	Content (%) of unlabeled and labeled rofecoxib				
	Unlabeled	$^{13}CH_3$	$^{13}CDH_2$	$^{13}CD_2H$	$^{13}CD_3$
A. [$^{13}CD_3$]Rofecoxib					
1. By GC–MS					
a. Neat standard in cyclooctane					>99.5
b. Neat standard in ACN	0.54	0.52	0.64	7.12	91.17
2. By HPLC–MS–MS					
a. Neat standard in ACN	0.24	17.33	7.15	9.66	65.62
b. Neat standard in ACN–water (50:50)	1.09	23.48	5.33	5.11	64.98
3. Spiked into human plasma, stored:					
a. 0 h; extracted	0.50	15.39	4.39	4.19	75.53
b. 3 h; extracted	0.27	15.28	4.22	4.43	75.80
c. 6 h; extracted	0.28	19.76	4.11	4.91	70.94
B. [$^{13}C_7$]Rofecoxib		$^{13}C_5$	$^{13}C_6$	$^{13}C_7$	
1. By GC–MS					
a. Neat standard in cyclooctane	0.0	0.2	5.6	94.2	
2. By HPLC–MS–MS					
a. Neat standard in ACN	0.0	0.24	5.81	93.95	
b. Neat standard in ACN–water (50:50)	0.0	0.23	5.43	94.34	
3. Spiked into human plasma, stored:					
a. 0 h; extracted	0.0	0.24	5.40	94.36	
b. 3 h; extracted	0.0	0.11	5.56	94.32	
c. 6 h; extracted	0.0	0.24	5.46	94.33	

[a] Values reported are averages of three to four replicate determinations.

and the mean value (PAR_n) for each subject was calculated. The ratio of **II** to **I** in i.v. dosing solutions for each subject was calculated by dividing PAR_n by PAR_s. Based on these ratios and the total content of both **I** and **II**, the individual concentrations of unlabeled and $^{13}C_7$-labeled drug in the i.v. dosing solutions of all subjects were calculated.

3. Results and discussion

3.1. Choice of stable-isotope-labeled rofecoxib

Dobson et al. [3] have listed four criteria for suitability of a stable-isotope labeled compound to be used in absolute BA studies. These criteria are as follows: (1) labeled drug must be distinguishable from the unlabeled drug using MS based methodology; (2) isotope labels must be sufficiently resistant towards exchange in the physiological and chemical environments; (3) label incorporation must be possible by a reasonable synthetic process; and (4) labeled drug must be pharmacokinetically indistinguishable from unlabeled drug.

Initially, [$^{13}CD_3$]rofecoxib was evaluated as the stable isotope labeled compound to be used in the BA study. A HPLC–MS–MS method was developed to distinguish the labeled from the unlabeled drug. Selected ion monitoring (SIM) and selected reaction monitoring (SRM) modes were used to determine the isotopic distribution of the labeled drug and both approaches gave similar results. Using SRM, the isotopic distribution was found to be 64.98% $^{13}CD_3$, 5.11% $^{13}CD_2H$, 5.33% $^{13}CDH_2$, 23.48% $^{13}CH_3$, and 1.09% of the unlabeled drug for standards prepared in acetonitrile–water (50:50) (Table 2, A.2.a). Similar distribution was observed when ACN was used as the solvent. However, the content of isotopic species was markedly different for the same material determined in a non-polar solvent (cyclooctane, Table 2, A.1.a) and ACN using GC–MS. Apparently, an efficient exchange of label was occurring in water containing solvents and/or in the HPLC mobile phase during HPLC–MS–MS analysis. Similar results obtained in ACN and ACN–water (1:1, v/v) may have indicated that the exchange has occurred during HPLC analysis although some hydrolysis in ACN was also occurring as indicated by the partial exchange observed in ACN vs. cyclooctane when monitored by GC–MS. This partial exchange in ACN is probably due to the presence of water in this solvent since no precautions were made to eliminate traces of water that is usually present in the ACN solvent. An isotopic exchange was also clearly observed after spiking [$^{13}CD_3$]rofecoxib into control plasma. A partial loss of label (deuterium) with time of incubation was observed (Table 2, A.3.a–c), although the degree of exchange in plasma was slightly less than in neat solvents. The isotopic exchange in ACN–water (1:1, v/v) was also confirmed by post-exchange extraction of [$^{13}CD_3$]rofecoxib with hexane and subsequent analysis by GC–MS and by the observed deuterium exchange between D_2O and unlabeled **I** (data not shown). The results of all these experiments clearly indicated that due to deuterium exchange in water containing solvents, [$^{13}CD_3$]rofecoxib was not suitable as an i.v. marker in the BA study.

Therefore, there was a clear need to synthesize a different stable-isotope-labeled-rofecoxib in which labels were sufficiently resistant towards exchange. Incorporation of seven ^{13}C atoms into the molecule (**II**, Fig. 2) gave a compound in which isotopic exchange was not observed. A similar set of experiments as performed for [$^{13}CD_3$]rofecoxib was repeated for [$^{13}C_7$]rofecoxib (**II**) and it was demonstrated that the content of $^{13}C_7$-label in the standards (Table 2, B.1.a–2 and B.a–b) and in plasma extracts (Table 2, B.3.a–c) was the same confirming the absence of an isotope exchange ''in vitro''. In addition, no ''cross-talk'' between the labeled **II** and the MS–MS channel used for monitoring the unlabeled **I** was observed. Compound **II** clearly met criterion 2 in Ref. [3] and was shown adequate to be utilized in the BA study.

3.2. Assay selectivity

The assay selectivity was assessed by analyzing extracts from five lots of plasma from different sources. Chromatograms of extracted control plasma and plasma spiked with **I**, **II**, and **III** are shown in Fig. 4. Endogenous peaks at the retention time of the analytes of interest were not observed in all plasma lots evaluated. All pre-dose plasma samples from subjects involved in the clinical study were also free

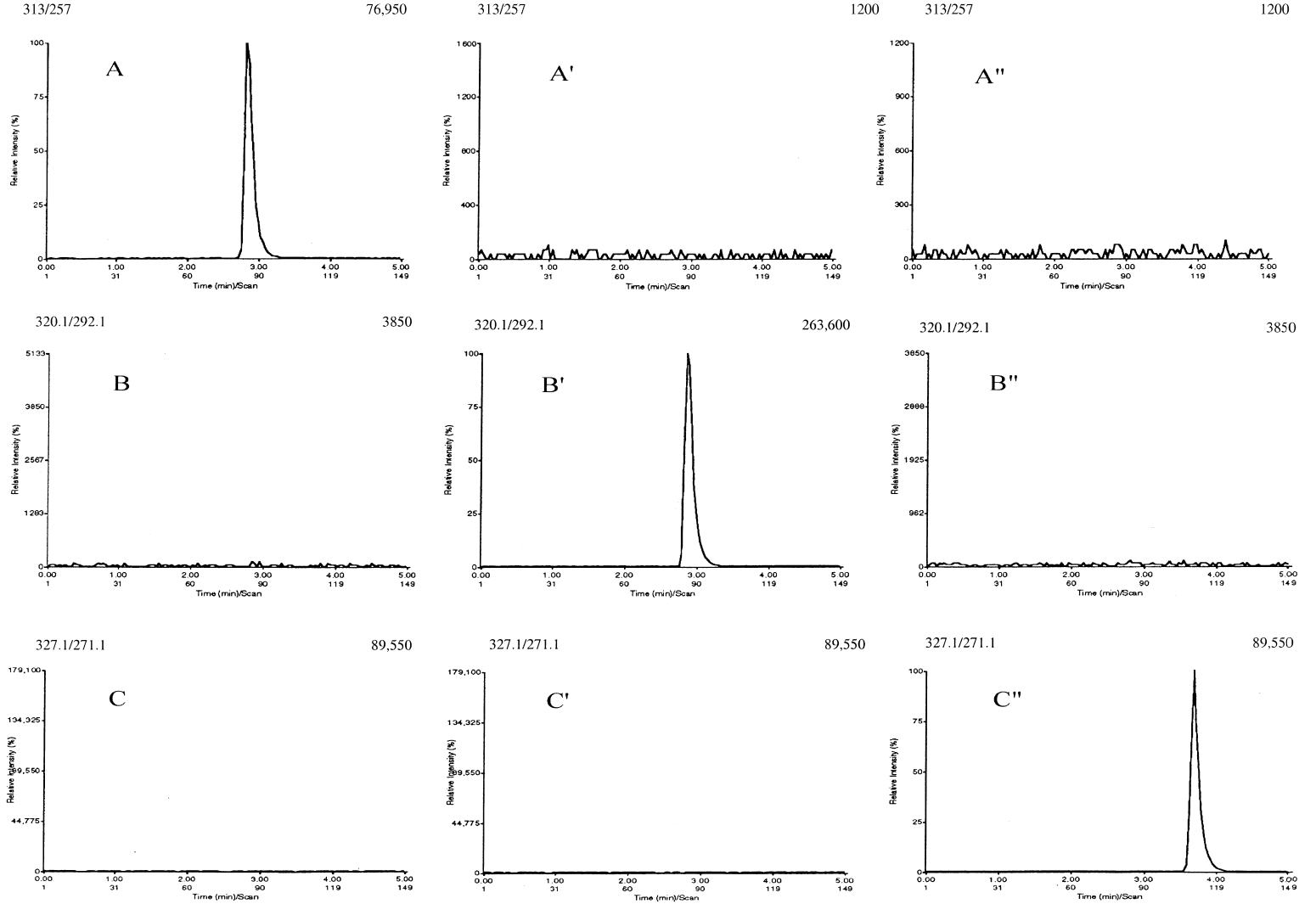


Fig. 4. Representative chromatograms of plasma extracts obtained by multiple reaction monitoring at m/z 313→257, for rofecoxib (**I**), m/z 320→292 for [$^{13}C_7$]rofecoxib (**II**), and m/z 327→271 for internal standard (**III**). Chromatograms A, B, and C – blank control plasma spiked with 100 ng/ml of **I**; chromatograms A′, B′, and C′ – control plasma spiked with 100 ng/ml of **II**; chromatograms A″, B″, and C″ – control plasma spiked with 100 ng/ml of **III**.

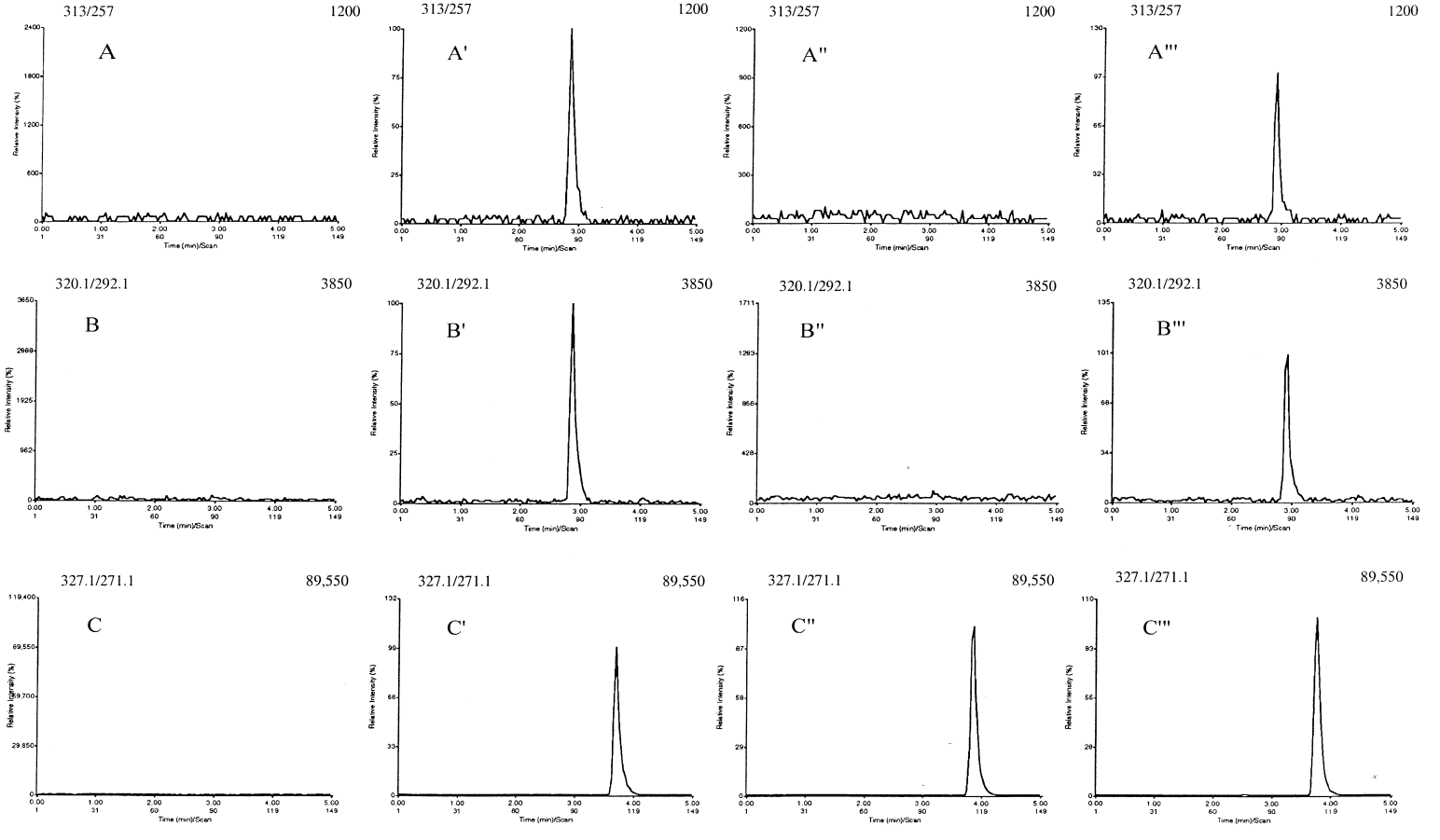


Fig. 5. Representative chromatograms of plasma extracts obtained by multiple reaction monitoring at m/z 313→257, for rofecoxib (**I**), m/z 320→292 for [$^{13}C_7$]rofecoxib (**II**), and m/z 327→271 for internal standard (**III**). Chromatograms A, B, and C – blank control plasma; chromatograms A′, B′, and C′ – control plasma spiked with 1.0 ng/ml each of **I** and **II** and 100 ng/ml of **III**; chromatograms A″, B″, and C″ – subject-004; 1 mg i.v. dose; pre-dose spiked with 100 ng/ml of **III**; chromatograms A‴, B‴, and C‴ – subject-004; 1 mg i.v. dose; 2.5 h post dose spiked with 100 ng/ml of **III** (concentrations of 0.50 and 0.58 ng/ml for **I** and **II**, respectively).

of interfering peaks. In addition, the ''cross-talk'' between MS–MS channels used for monitoring **I**, **II**, and **III**, at the highest concentrations of each analytes, was not observed (Fig. 5).

3.3. Linearity

The linearity of standard curve was confirmed by plotting the peak area ratio of the drug to I.S. vs. drug concentration. The unknown sample concentrations were calculated from the equation $y=mx+b$, as determined by weighted $(1/y^2)$ linear regression analysis of the standard line. Typical equations describing calibration lines were $y=0.01658x+0.000104$ for **I** and $y=0.049559x+0.001649$ for **II** with correlation coefficients of >0.99.

3.4. Assay precision and accuracy

The precision of the method was determined by the replicate analyses ($n=5$) of human plasma containing **I** and **II** at all concentrations utilized for the construction of calibration curves. The accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)]·100. The precision of the assay, expressed as relative standard deviation (RSD), was less than 10% at all concentrations within the standard curve range with adequate assay accuracy (Table 3). The inter-day variability, as measured by the assay of QC samples, was less than 7% for both analytes (Table 4).

3.5. Bioavailability study of rofecoxib

The HPLC–MS–MS assay was utilized to support a clinical study to determine bioavailability of 12.5- and 25-mg oral doses of rofecoxib, using i.v. doses of $^{13}C_7$-labeled rofecoxib. In the first part of the study, the pharmacokinetic equivalence of unlabeled and labeled rofecoxib was verified (criterion 4 [3]). In this period, nine subjects received a single i.v. dose of labeled and unlabeled rofecoxib (1 mg each), and the concentrations of both analogs in plasma were determined at pre-dose to 73 h after dosing. It was necessary to compare the ratio of the area under the curves (AUCs) of labeled to unlabeled drug to the measured ratio in the actual i.v. solutions administered. The ratios of **II** to **I** in the i.v. dosing solutions were determined as described in Section 2.9. The isotope ratios observed in the dosing solution and the ratios of the AUCs were comparable which indicated the absence of an isotope effect on the rates of elimination of the drug. Therefore, [$^{13}C_7$]rofecoxib was a suitable isotope-labeled reference standard for BA studies meeting criterion 4 in Ref. [3].

In the second part of the study, subjects received 12.5- or 25-mg oral doses of **I** while they simul-

Table 3
Precision and accuracy of replicate analysis ($n=5$) of rofecoxib and [$^{13}C_7$]rofecoxib in human plasma

Nominal concentration (ng/ml)	Rofecoxib			[$^{13}C_7$]Rofecoxib		
	Mean[a] concentration (ng/ml)	Precision[b]	Accuracy[c] (%)	Mean[a] concentration (ng/ml)	Precision[b]	Accuracy[c] (%)
0.10	0.10	5.3	100.0	0.10	4.7	100.0
0.25	0.26	8.7	104.0	0.25	4.4	100.0
0.50	0.48	4.9	96.0	0.52	4.2	104.0
1.00	1.01	3.0	101.0	1.03	3.6	103.0
5.00	5.06	1.1	101.2	5.13	8.4	102.6
10.00	10.02	3.3	100.2	10.01	3.8	100.1
50.00	49.26	3.3	98.5	48.93	5.5	97.9
100.00	100.62	1.1	100.6	100.62	1.1	100.6

[a] Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as relative standard deviation (RSD, %).
[c] Expressed as [(mean calculated concentration)/(nominal concentration)·100].

Table 4
Analysis of plasma quality control samples for rofecoxib and [$^{13}C_7$]rofecoxib concentrations

	Rofecoxib		[$^{13}C_7$]Rofecoxib	
	LQC[a] concentration (ng/ml)	HQC[b] concentration (ng/ml)	LQC[a] concentration (ng/ml)	HQC[b] concentration (ng/ml)
Nominal concentration:	0.75	75.0	0.75	75.0
Initial mean (n=5)				
Assayed				
Concentration[c]	0.74	79.95	0.80	74.24
SD[d]	0.03	3.9	0.01	3.6
RSD (%)	4.5	4.9	1.2	4.9
Inter-day variability[e]				
Overall mean (n=14)	0.77	77.66	0.76	74.53
SD	0.03	4.43	0.04	4.80
RSD (%)	4.3	5.7	5.3	6.4

[a] Low quality control.
[b] High quality control.
[c] Mean of n=5.
[d] Standard deviation.
[e] Over a period of 11 days.

taneously received an i.v. dose of **II** (1 mg). A representative plasma vs. time profile for i.v. and oral drug following coadministration of 1 mg **II** (i.v.) and 12.5 mg **I** (oral) to a subject is shown in Fig. 6. Bioavailability of the 12.5 and 25 mg formulations of rofecoxib was estimated using the results of the analyses of plasma samples collected from the second part of this study. A thorough discussion of the pharmacokinetic results of the BA study will be reported separately [14].

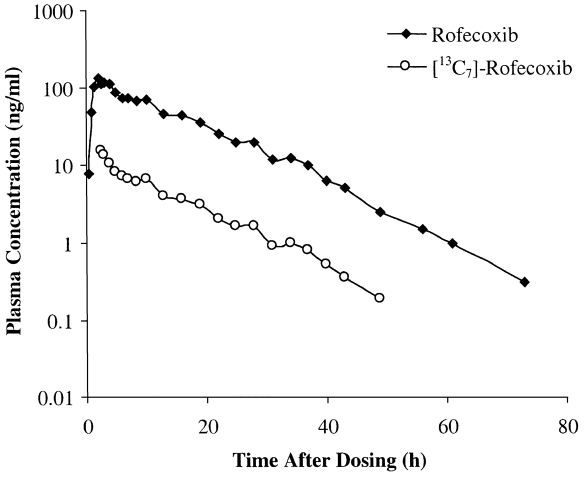


Fig. 6. A representative plasma vs. time profile for **I** and **II** in plasma from a human subject following coadministration of 12.5 mg **I** (orally) and 1 mg **II** (i.v.).

4. Conclusions

The results presented in this paper clearly demonstrate the need for careful evaluation and a proper choice of stable isotope-labeled drugs that are used in metabolism and BA studies. The absence of an isotope exchange under the experimental conditions used in these studies and during the analyses needs to be confirmed. In addition, the absence of an isotope effect on the rate of disposition of the labeled vs. unlabeled analogs and the absence of a ''cross-talk'' between MS–MS channels used for monitoring the unlabeled and labeled analyte at widely different concentrations needs to be demonstrated. It was shown that [$^{13}CD_3$]rofecoxib was not suitable for BA studies due to an efficient deuterium exchange in water containing solvents and in plasma. Instead, [$^{13}C_7$]rofecoxib was demonstrated to meet all the analytical criteria necessary for conducting studies ''in vivo''. Analytical techniques required for assessing the suitability of isotope-labeled analogs in clinical studies utilizing the HPLC–MS–MS and other techniques were presented.

A sensitive and selective HPLC–MS–MS method for the simultaneous determination of rofecoxib and [$^{13}C_7$]rofecoxib has been developed and this method was successfully utilized for the analysis of plasma samples from a clinical study that resulted in the determination of the oral BA of rofecoxib.

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