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High-performance liquid chromatographic determination of the stereoisomeric metabolites of ibuprofen

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SUMMARY

A stereospecific reversed-phase high-performance liquid chromatographic (HPLC) method has been developed to simultaneously quantitate the stereoisomers of the two major metabolites of ibuprofen: hydroxyibuprofen and carboxyibuprofen. The metabolites were derivatized with *S*-(α)-methylbenzylamine to form diastereomeric amides which were separated and quantified on a C_8 column. The validity of the stereoselective assay was confirmed by comparison with a non-stereoselective HPLC method. The stereoselective assay was applied to the quantification of all the stereoisomeric ibuprofen metabolites in urine from human volunteers dosed with racemic ibuprofen or the individual enantiomers of ibuprofen. Significant substrate and product stereoselectivities were observed in the formation of carboxyibuprofen.

INTRODUCTION

In humans, ibuprofen is eliminated from the body principally via the formation of 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (hydroxyibuprofen) and 2-[4-(2-carboxypropyl)phenyl]propionic acid (carboxyibuprofen). Following oral administration of ibuprofen, approximately 28 and 40% of the dose is recovered in urine as the hydroxy and carboxy metabolites, respectively, as a mixture of conjugated and unconjugated forms [1–3]. Like ibuprofen, hydroxyibuprofen exists as a pair of enantiomers. In contrast, the formation of carboxyibuprofen introduces a second chiral center; therefore,

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four stereoisomeric products are possible, that is *RS*- (*R*-propionic acid, *S*-carboxy), *SR*-, *SS*-, and *RR*-configurations [4].

When racemic ibuprofen was administered to humans, both the hydroxy and carboxy metabolites excreted in urine were found to be dextrorotatory primarily as a result of the unusual *R*-(−)- to *S*-(+)-configurational inversion known to occur for some arylpropionic acids, including ibuprofen [2,5,6]. Subsequently, Kaiser et al. [4] indicated that all the possible stereoisomeric hydroxy and carboxy metabolites are present in human urine following ibuprofen dosing. Despite significant interest in this area, the complete description of the relative rates of formation of these metabolites has not been forthcoming due to the lack of a completely stereoselective assay. Previously, the most successful approach to this problem has been the formation of diastereomeric amide derivatives with *S*-(α)-methylbenzylamine and resolution of the products via gas chromatography using packed or capillary columns [3,4,7]. Unfortunately, the latter approaches fail to resolve all of the stereoisomeric carboxy metabolites. Nicoll-Griffith et al. [8] examined the ability of a Pirkle column to resolve the 4-methoxyanilide derivatives of ibuprofen and its metabolites but were also unable to quantitate the individual metabolite stereoisomers, particularly for carboxyibuprofen.

We, and others, have previously noted that reversed-phase high-performance liquid chromatography (HPLC) is associated with good resolution of the *S*-(α)-methylbenzylamide derivatives of arylpropionic acids [9] and their metabolites as illustrated in the case of flurbiprofen [10]. In this report, we utilize this approach to resolve all the stereoisomeric hydroxy and carboxy metabolites of ibuprofen and illustrate its utility to define the metabolite excretion profile of ibuprofen.

EXPERIMENTAL

Materials

Racemic hydroxyibuprofen and carboxyibuprofen were generously supplied by Upjohn (Kalamazoo, MI, U.S.A.) and were used without further purification. β-Naphthoic acid was purchased from Sigma (St. Louis, MO, U.S.A.) and 5-ethyl-5-*p*-tolylbarbituric acid (ETBA), (*S*)-(−)-α-methylbenzylamine, and 1,1′-carbonyldiimidazole (CDI) were purchased from Aldrich (Milwaukee, WI, U.S.A.). The (*S*)-(−)-α-methylbenzylamine obtained from this source has been shown to be essentially free of optical impurity by HPLC [11]. Organic solvents (Fisher Scientific, Fair Lawn, NJ, U.S.A.) were HPLC grade with the exceptions of chloroform (ACS grade) and acetone (NF grade). Acetic acid, ammonium hydroxide, phosphoric acid, and hydrochloric acid were ACS grade (Fisher Scientific).

Apparatus and chromatographic conditions

In addition to a stereospecific procedure (assay I) a non-stereoselective procedure (assay II), based on that of Shah and Jung [12], was developed to evaluate the accuracy of assay I.

Assay I. HPLC was performed on a system containing a Beckman Model 114M pump (Beckman, San Ramon, CA, U.S.A.), a Spectra-Physics Model SP8780 autosampler (Spectra Physics, San Jose, CA, U.S.A.) fitted with a 200-μl loop, and a BAS Model UV-116 variable-wavelength UV detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) set at 232 nm.

Separation of stereoisomers was achieved on an Ultrasphere octyl column (5 μm particle size, 25 cm $\times$ 4.6 mm I.D.) (Beckman) protected by a 1.5 cm $\times$ 3.2 mm I.D. octyl guard column (RP8, Brownlee Labs., Santa Clara, CA, U.S.A.) with a mobile phase of water–methanol–*n*-butanol (62:30:8, v/v) at a flow-rate of 1 ml/min.

Assay II. Instrumentation was identical to that outlined for assay I above with the following exceptions. Non-stereoselective separation of the hydroxy and carboxy metabolites was achieved with a Beckman Ultrasphere ODS column (5 μm, 25 cm $\times$ 4.6 mm) using a mobile phase of water–acetonitrile (74:26, v/v) with 0.5 ml each of acetone and phosphoric acid per liter of mobile phase at a flow-rate of 1 ml/min.

Sample preparation

Assay I. The hydrolysis of conjugated metabolites was performed by adding 1 ml of urine to an equal volume of 6 *M* hydrochloric acid followed by incubation in a 90 °C water bath for 30 min. Preliminary studies revealed that deconjugation was maximal and negligible amounts of the hydroxy and carboxy metabolites were lost during this procedure. Acid hydrolysis was employed to ensure cleavage of ether and ester glucuronides [8,13]. After a 10-min cooling period and the addition of 40 μl of internal standard (β-naphthoic acid, 74 μg/ml in methanol) each sample was extracted twice with 7 ml of methylene chloride by shaking for 20 min. This double extraction was important to enhance the extraction efficiency and reproducibility. The samples were centrifuged for 4 min at 800 *g* between extractions. The organic phases for each sample were combined and the solvent evaporated to dryness in a 37 °C water bath under a stream of nitrogen. Calibration standards over the range 6–50 μg/ml of the individual isomers were prepared in water and processed in an identical fashion. Preliminary studies indicated that water and blank urine gave the same results with respect to the construction of calibration lines.

The samples were derivatized by a modification [10] of the procedure of Maître et al. [9]. The sample residues were reconstituted with 300 μl of chloroform. A 200-μl aliquot of CDI (65 mg/ml in chloroform, prepared fresh daily) was added to each sample, gently mixed and allowed to stand at room temperature for 10 min to form the imidazolide intermediates [14]. Glacial acetic acid

(10 μl) was added and the samples were allowed to react for 10 min. A 50-μl volume of the derivatizing agent *S*-(−)-(α)-methylbenzylamine was added and after mixing, samples were allowed to react for 30 min to form the diastereomeric amides. The reaction mixture was washed by addition of 3 ml of 0.5 *M* ammonium hydroxide. Subsequently hexane (5 ml) was added to each tube, the samples were rocked for 15 min, and the phases separated by centrifugation. The chloroform–hexane layer was removed and similarly washed with 3 ml of 1 *M* hydrochloric acid. The organic layer containing derivatized ibuprofen metabolites was evaporated to dryness under nitrogen, reconstituted with 250 μl of water–methanol (62:38, v/v), and a 30-μl aliquot was injected onto the HPLC system.

Assay II. For the non-stereoselective assay of hydroxy and carboxy metabolites in urine, 0.5-ml aliquots of thawed urine were hydrolyzed as described for assay I. After hydrolysis, 50 μl of internal standard (ETBA, 650 μg/ml in methanol) were added and samples were extracted once with 6 ml of methylene chloride by shaking for 20 min. After centrifugation the aqueous layer was aspirated to waste and the organic phase was transferred to a new tube. The solvent was evaporated to dryness under nitrogen in a 37°C water bath and the sample was reconstituted with 0.5 ml of mobile phase. A 20-μl aliquot of each sample was injected. Standard curve samples with concentrations from 20 to 250 μg/ml for each metabolite were prepared in water and processed as for samples.

Data analysis

A five-point standard curve and appropriate controls were analyzed with each set of samples. Metabolite quantitation was based on least-squares linear regression analysis of peak-height ratio versus concentration. The accuracies of assays I and II were assessed by calculating the relative error (R.E.) associated with samples of known concentration. R.E. was obtained by dividing the actual minus observed concentration by the actual concentration and expressing as a percentage. Assay precision was estimated via the coefficient of variation (C.V.) associated with replicate samples run on the same day (intra-day) and different days (inter-day). The C.V., expressed as a percentage, was determined by dividing the standard deviation by the mean value. Statistical comparisons were performed by ANOVA followed by the Student-Newman-Keuls test ($p<0.05$) using PC-SAS (Version 6, SAS Institute, Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

Derivatization of racemic arylpropionic acids and their metabolites with *S*-(α)-methylbenzylamine has previously been employed to quantitate these compounds on common achiral columns [9,10]. We have extended this ap-

proach to resolve and quantify all the stereoisomeric metabolites of ibuprofen (assay I) and have verified the accuracy of this assay by comparing it to a non-stereoselective procedure (assay II).

Assay I

Fig. 1A illustrates that pre-dose blank urine was essentially free of interfering peaks, a feature common to all subjects studied to date. The elution order of the metabolites was determined by analyzing the urine from a human subject who had received a dose of pure *S*-ibuprofen. *S*-Ibuprofen is not inverted to the *R*-enantiomer [6,15] and therefore when *S*-ibuprofen (600 mg) is administered the peaks corresponding to the *S*-metabolites, that is *S*-hydroxyibuprofen, *SS*- and *SR*-carboxyibuprofen, are readily identified (Fig. 1D). Examination of Fig. 1B and C illustrates that following administration of *R*-ibuprofen (600 mg) or racemic ibuprofen (800 mg) all peaks of interest are well resolved with a peak resolution factor of 1.0 or greater for adjacent peaks. The retention times for *S*- and *R*-hydroxyibuprofen, internal standard, *SS*-, *RS*-, *SR*-, and *RR*-carboxyibuprofen were 17, 19, 41, 44, 52, 55, and 64 min, respectively. To our knowledge, reference standards of the carboxy metabolite isomers are not available and therefore we cannot unequivocally assign the elution order of *SS*- and *SR*- or *RS*- and *RR*-carboxyibuprofen. However, for consistency we have reproduced the gas chromatographic method of Kaiser et al. [4] to confirm that the peaks these workers designated as the *SS*- and *SR*-isomers correspond to these isomers in our system.

Calibration lines for all stereoisomeric metabolites over the range 6–50 μg/ml were linear ($r > 0.99$). Below 6 μg/ml the C.V. often exceeded 10% and hence this was set as the limit of detection. Under the conditions studied this sensitivity was sufficient to characterize the excretion of all the stereoisomeric metabolites. Table I indicates that the intra-day and inter-day C.V.s and R.E.s were less than 8% and that the assay performed satisfactorily.

Assay II

The chromatographic conditions for this assay were modified from those previously reported [12] to ensure that an interfering peak (peak 2 in Fig. 2B) was completely resolved from carboxyibuprofen. Blank urine obtained prior to drug administration was free from interfering peaks (Fig. 2A) for all subjects. The retention times of hydroxyibuprofen, carboxyibuprofen, and the internal standard were 13.4, 17.7, and 20.3 min. Standard curves were linear for both metabolites over the range 20–250 μg/ml ($r > 0.99$). This assay also exhibited good accuracy and reproducibility as indicated in Table II.

Comparison of assays I and II

The agreement between results obtained for assay I and assay II was examined by adding together stereoisomeric hydroxy and carboxy concentrations to

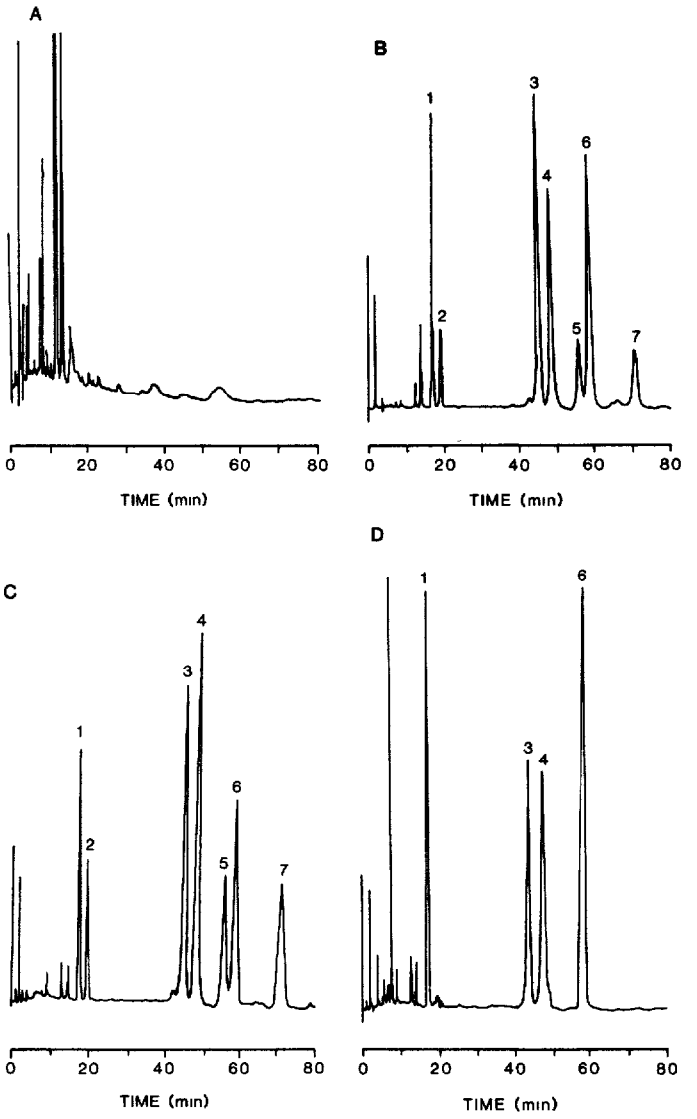

Fig. 1. Chromatograms of the diastereomeric amide derivatives of ibuprofen metabolites extracted from human urine from a healthy volunteer. (A) Pre-dose blank urine (0.01 a.u.f.s.); (B), (C) and (D) urines from the 240–360 min collection period from a subject dosed with 800 mg racemate, 600 mg *R*-ibuprofen and 600 mg *S*-ibuprofen, respectively (0.02 a.u.f.s. for 40 min then 0.16 a.u.f.s.). Peaks: 1 = *S*-hydroxyibuprofen; 2 = *R*-hydroxyibuprofen; 3 = internal standard; 4 = *SS*-carboxyibuprofen; 5 = *RS*-carboxyibuprofen; 6 = *SR*-carboxyibuprofen; 7 = *RR*-carboxyibuprofen.

give total hydroxy and carboxy concentrations and comparing these totals to the results of assay II. For 21 urine samples, which spanned the concentration

TABLE I

INTRA-DAY AND INTER-DAY REPRODUCIBILITY AND ACCURACY OF REPLICATE ANALYSES FOR ASSAY I

Compound	Intra-day[a] ($n=3$)		Inter-day[a] ($n=4$)		Accuracy ($n=8$)	
	Mean concentration (μg/ml)	C.V. (%)	Mean concentration (μg/ml)	C.V. (%)	R.E. (%)	C.V. (%)
S-Hydroxyibuprofen	40.1	3.9	39.8	3.5	8.0	3.8
R-Hydroxyibuprofen	9.6	4.6	9.6	3.7	6.2	4.6
SS-Carboxyibuprofen	43.3	6.1	43.6	5.2	0.6	7.1
RS-Carboxyibuprofen	7.4	7.2	7.2	7.1	4.8	5.5
SR-Carboxyibuprofen	31.1	5.9	30.8	5.4	6.8	7.1
RR-Carboxyibuprofen	10.5	6.0	10.4	5.4	6.8	6.1

[a]Urine obtained following racemic ibuprofen administration.

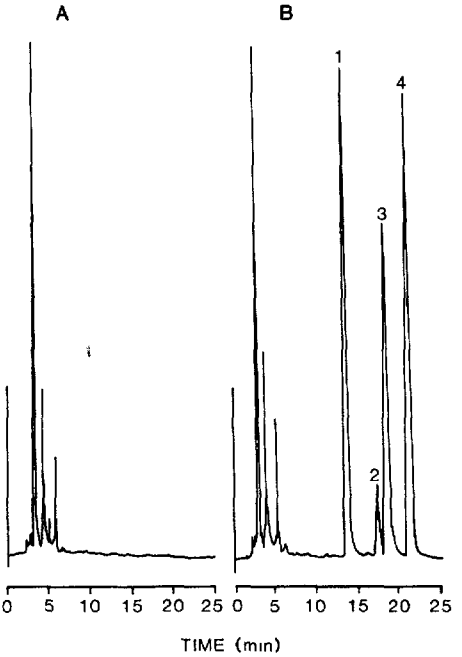

Fig. 2. Chromatograms of ibuprofen metabolites extracted from urine. (A) Pre-dose blank (0.05 a.u.f.s.); (B) urine from the collection period 120–160 min from a subject dosed with 600 mg of *S*-ibuprofen (0.05 a.u.f.s.). Peaks: 1 = hydroxyibuprofen; 2 = unknown; 3 = carboxyibuprofen; 4 = internal standard.

TABLE II

INTRA-DAY AND INTER-DAY REPRODUCIBILITY AND ACCURACY OF REPLICATE ANALYSES FOR ASSAY II

Compound	Intra-day[a] ($n=8$)		Inter-day[a] ($n=4$)		Accuracy ($n=8$)	
	Mean concentration (μg/ml)	C.V. (%)	Mean concentration (μg/ml)	C.V. (%)	R.E. (%)	C.V. (%)
Hydroxyibuprofen	25.8	1.5	23.6	7.1	−4.7	1.5
Carboxyibuprofen	43.1	1.0	35.3	4.2	−0.33	1.0

[a]Urine obtained following racemic ibuprofen administration.

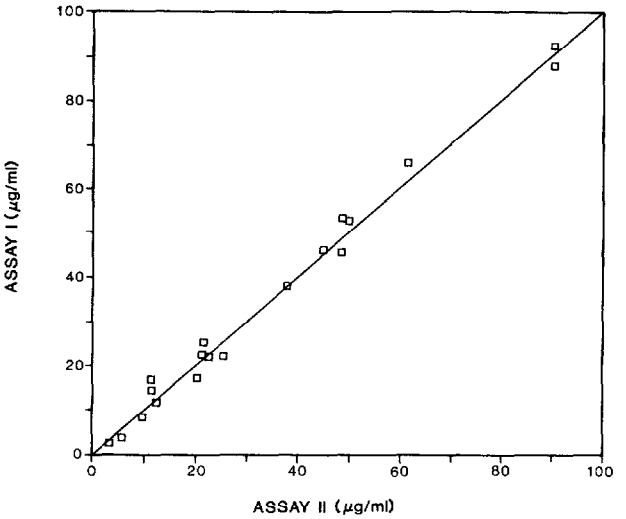

Fig. 3. Comparison of assays I and II for hydroxyibuprofen. Samples represent the urine concentrations for sequential urine samples obtained 0–22 h after doses of racemic (800 mg) and *R*-ibuprofen (600 mg) administered to a representative individual ($n=21$). Linear regression estimates a slope of 1.02 ± 0.024; $r=0.995$.

range of interest, there was good correlation between the predictions of these two assays for both the hydroxy (Fig. 3) and carboxy (Fig. 4) metabolites.

Application

The utility of this stereoselective assay was examined by the analysis of 0–22 h urine samples obtained from six normal subjects who received racemic ibuprofen (800 mg), *R*-ibuprofen (600 mg) or *S*-ibuprofen (600 mg) orally in

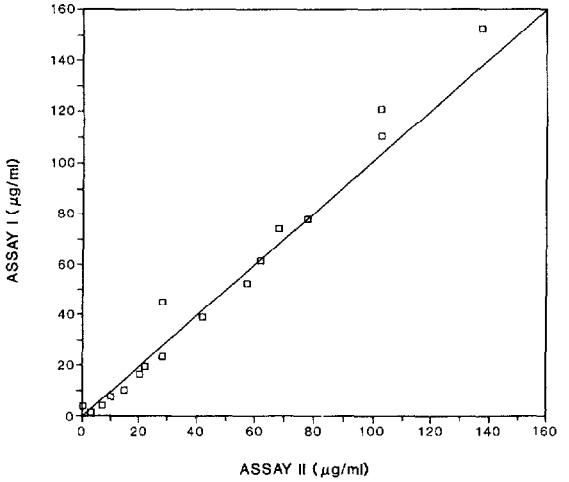

Fig. 4. Comparison of assays I and II for carboxyibuprofen. Samples are identical to Fig. 3 ($n=21$). Linear regression estimates a slope of 1.03 ± 0.025; $r=0.995$.

random order. Total urinary recovery, expressed as a percentage of the administered dose (mean $\pm$ S.D.) of hydroxyibuprofen ($21.8 \pm 3.9\%$) and carboxyibuprofen ($36.4 \pm 7.1\%$) following the racemic dose is in good agreement with previous reports [1,2,16]. Similarly, following administration of *R*-ibuprofen our recovery of total hydroxyibuprofen ($21.7 \pm 4.7\%$) and carboxyibuprofen ($33.4 \pm 7.0\%$) corresponds closely to that reported by Baillie et al. [3]. When the dose of *S*-ibuprofen is considered, the total recovery of these metabolites was 28.3 ± 4.8 and $43.3 \pm 5.0\%$, respectively. Interestingly, the recovery of the carboxy but not the hydroxy metabolite is significantly greater ($p<0.05$) for the *S*-dose relative to the others implying that a significant substrate stereoselectivity exists for the formation of carboxyibuprofen.

In agreement with previous studies the amount of *S*-hydroxyibuprofen in urine exceeded that of the *R*-isomer after all ibuprofen doses (Table III). Following oral doses of racemate and *R*-ibuprofen 79 and 63% of the hydroxyibuprofen was excreted in the *S*-configuration which is comparable to the results of Kaiser et al. [4] who found 71 and 54%, respectively, and Baillie et al. [3] who found 62% following administration of *R*-ibuprofen. Similarly, urinary carboxyibuprofen was enriched in metabolites derived from *S*-ibuprofen, that is *SS*- and *SR*-carboxyibuprofen (Table III). This enrichment in the *S*-acid configuration for both hydroxy- and carboxyibuprofen probably reflects unidirectional chiral inversion of *R*-ibuprofen and subsequent metabolism [6,17].

Previous studies have not been able to resolve the peaks designated as *SR*- and *RS*-carboxyibuprofen [3,4,7,8]. Our data indicate that there is a substantial difference in the excretion rates of these two metabolites and therefore

TABLE III

STEREOISOMERIC COMPOSITION OF IBUPROFEN METABOLITES FOUND IN HUMAN URINE AFTER SINGLE DOSES OF RACEMATE (800 mg), *R*-IBUPROFEN (600 mg), OR *S*-IBUPROFEN (600 mg)

Values are percentage of dose on a molar basis, recovered in six subjects (mean ± S.D.).

Dose	Hydroxyibuprofen		Carboxyibuprofen			
	S	*R*	*SS*	*RS*	*SR*	*RR*
Racemate	17.1 ± 3.0	4.6 ± 1.2	14.9 ± 3.5	2.8 ± 0.92	15.1 ± 4.1	3.5 ± 1.3
R-Ibuprofen	13.5 ± 2.3	8.2 ± 2.4	11.8 ± 2.2	4.8 ± 1 7	9.9 ± 1.7	6.8 ± 2.2
S-Ibuprofen	26.6 ± 4.8	1.7 ± 0.6	18.3 ± 2.9	0.8 ± 1.4	24.1 ± 6.6	0.08 ± 0.15

TABLE IV

URINARY DIASTEREOMERIC RATIOS FOR CARBOXYIBUPROFEN FOLLOWING ADMINISTRATION OF *R*-, RACEMIC OR *S*-IBUPROFEN

Values are ratios of amounts recovered (mean ± S.D.) in six human subjects.

Dose	*SS*/*SR* ratio	*RS*/*RR* ratio[a]
R-Ibuprofen	1.19 ± 0.12	0.71 ± 0.13
Racemate	1.05 ± 0.36	0.83 ± 0.23
S-Ibuprofen	0.83 ± 0.34	–[b]

[a]Significant difference between *R* and racemate ($p < 0.05$).
[b]Not calculated because metabolite concentrations were below the limit of detection.

erroneous conclusions may be reached if they are assumed to be equal [3]. The diastereomeric ratios (*SS*/*SR* and *RS*/*RR*) for carboxyibuprofen are presented in Table IV. No significant differences in excretion rate, due to the mode of administration, were detected for *SS*- and *SR*-carboxyibuprofen but there was a wide range of *SS*/*SR* ratios. However, the *RS*/*RR* ratio was significantly greater following administration of racemate relative to *R*-ibuprofen alone implying that there is a modest product stereoselectivity favoring the formation of the *RS*-isomer.

CONCLUSIONS

A reproducible and accurate reversed-phase HPLC method is presented which allows the simultaneous assay of all the stereoisomers of hydroxy- and carboxyibuprofen. The method was employed to study the stereoselective formation of the major metabolites of ibuprofen and revealed significant substrate and product stereoselectivities.

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