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Stereospecific analysis of the major metabolites of ibuprofen in urine by sequential achiral–chiral high-performance liquid chromatography

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Abstract

A sequential achiral–chiral HPLC method has been developed for the stereospecific analysis of the two major urinary metabolites of ibuprofen, namely hydroxyibuprofen and carboxyibuprofen. Achiral analysis was carried out using a Partisil column (250×4.6 mm, 5 μm) and a mobile phase of hexane:ethanol (98.2:1.8, v/v) containing trifluoroacetic acid (TFA; 0.05%, v/v) at a flow-rate of 2.0 ml/min. The HPLC eluate containing the two metabolites was separately collected, evaporated under nitrogen and the residue dissolved in the mobile phase used for chiral chromatography. Chiral-phase analysis was carried out using a Chiralpak AD CSP (250×4.6 mm, 10 μm) with a mobile phase of hexane:ethanol (92:8, v/v) containing TFA (0.05%, v/v) at a flow-rate of 1.0 ml/min. In both assays the analytes were quantified by ultraviolet detection at a wavelength of 220 nm. Modification of the mobile-phase composition allowed the resolution of all six analytes in a single chromatographic run but with an increase in run time and consequent band broadening. The analytical method described allows the direct quantitation of the stereoisomers of both metabolites of ibuprofen in urine following the administration of therapeutic doses of the racemic drug to man. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Ibuprofen; Hydroxyibuprofen; Carboxyibuprofen

1. Introduction

(*R,S*)-Ibuprofen [(±)-(*R,S*)-2-(4-isobutylphenyl)propionic acid], a nonsteroidal antiinflammatory drug (NSAID) widely used for the treatment of pain and inflammation in rheumatic disease and other musculoskeletal disorders, is with the exception of Austria marketed as a racemate. Similarly to other members of the 2-arylpropionic acid (2-APA) group of NSAIDs, ibuprofen shows stereoselectivity in both action and disposition [1–5] and was the first compound of this group reported to undergo chiral inversion from the inactive *R*-enantiomer to its active *S*-antipode [6,7]. In addition to chiral inversion, the metabolism of ibuprofen involves conjugation with glucuronic acid and oxidation to yield two major products, hydroxyibuprofen (2-[4- (2-hydroxy-2-methylpropyl)phenyl]propionic acid) and carboxyibuprofen (2-[4-(2-carboxypropyl)phenyl]propionic acid) [8,9] (Fig. 1). The urinary excretion of these two metabolites and the unchanged drug, both free and conjugated with

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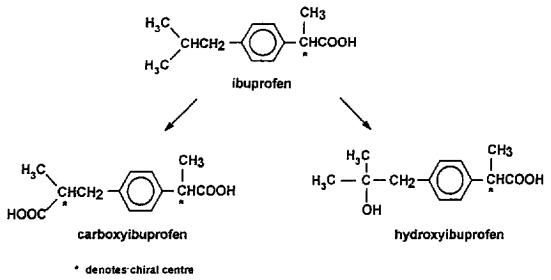

Fig. 1. Major oxidative metabolic pathways of ibuprofen.

glucuronic acid, accounts for approximately 80% of an oral dose following administration of racemic ibuprofen to man [10,11]. The observation that both metabolites were dextrorotatory irrespective of the stereoisomeric form in which the drug was administered, i.e. either individual enantiomer or racemate, resulted in the discovery of the chiral inversion reaction for this series of NSAIDs [2,3,8,9].

As a result of the interest in the stereochemical aspects of its metabolism, together with the possible implications of the chiral inversion reaction, the metabolism of ibuprofen has been extensively investigated both in vivo and in vitro [2–5]. However, in spite of the intense interest in the stereochemical aspects of the metabolism and pharmacokinetics of the parent drug, the stereoselective disposition of the two major oxidation products has rarely been studied. In addition, the achiral chromatographic analysis of the two major metabolites of ibuprofen in biological fluids has received relatively little attention in the literature [12–17], and the stereospecific analysis has proved to be problematic. The chromatographic resolution of (*R*)- and (*S*)-hydroxyibuprofen has been achieved by a variety of methods based on the indirect approach to enantiomeric analysis [7,18–22] and also by the direct approach using a Pirkle type chiral stationary phase (CSP), (*R*)-*N*-(3,5-dinitrobenzoyl)phenylglycine [23]. However, the metabolic formation of carboxyibuprofen results in the introduction of a second chiral centre in the molecule and, therefore, following the administration of either racemic or (*R*)-ibuprofen, four stereoisomers are possible. Neither the original packed column GLC assay [7] or the more recent capillary GC–MS assays [18,19] based on the indirect approach have resulted in the resolution of all four stereoisomers, coelution of two of the stereoisomers being observed in each case. The application of the above Pirkle CSP to the resolution resulted in the separation of the pairs of diastereoisomers but not in enantiomeric resolution [23]. The only reported chromatographic resolution of the stereoisomers of carboxyibuprofen is that of Rudy et al. [20], which is based on the indirect approach following derivatization with (*S*)-1-phenylethylamine and high-performance liquid chromatography (HPLC) using a C_8 stationary phase. The lack of readily available, stereochemically defined, authentic standards has also resulted in problems associated with the determination of the chromatographic elution order. The stereochemical assignment of the chromatographic peaks being based [19,20] on that of Kaiser et al. [7], which was empirical. The (*S*)-1-phenylethylamine derivatives of (*S*)-ibuprofen and hydroxyibuprofen eluting before those of the *R*-configuration resulting in the deduction that the corresponding diamide derivatives of the diacid metabolite would elute in the order *S,S* before *R,S*/*S,R* before *R,R* [7].

We have recently reported the chromatographic resolution, chiroptical properties and, by a combination of stereoselective synthesis and metabolic approaches, determined the chromatographic elution order of the stereoisomers of carboxyibuprofen on a derivatized amylose CSP, amylose tris(3,5-dimethylphenylcarbamate) (Chiralpak AD) [24]. We have now extended these investigations to develop a sequential achiral–chiral chromatographic technique, based on the Chiralpak AD CSP, for the determination of the stereochemical composition of hydroxy and carboxyibuprofen in human urine. Total metabolite concentrations are determined by achiral normal-phase chromatography, followed by collection of the column eluates containing the individual metabolites prior to the determination of their stereochemical composition using the CSP. Carboxyibuprofen is chemically designated as 2-[4-(2-carboxypropyl)phenyl]propionic acid. Using this nomenclature, both chiral centres are indicated as being at the two position of the two side chains. For the purpose of clarity in this report, the chiral centre in the propionic acid moiety of ibuprofen is designated as the 2-position, whereas that introduced by metabolic oxidation of the isobutyl group is designated as the 2′-position.

2. Experimental

2.1. Chemicals

(*R*)-, (*S*)- and (*R,S*)-Ibuprofen and hydroxyibuprofen were the generous gifts of Boots Co. Ltd. (Nottingham, UK), 'racemic' carboxyibuprofen was synthesized as described previously [24]. Dichloromethane, ethanol, ethyl acetate and hexane (HPLC grade) were purchased from Rathburn (Walkerburn, UK). Trifluoroacetic acid, sodium dihydrogen phosphate, sodium hydroxide (Analar grade) and other organic solvents (GPR grade) were obtained from BDH (Poole, UK). Hexamethyldisilazane and 4-chlorophenoxyacetic acid were obtained from Sigma Chemicals (Poole, UK).

2.2. Chromatographic columns and supplies

The Partisil silica column (250×4.6 mm, 5 μm) was obtained from Whatman (Maidstone, UK). Refillable guard columns (10×2.1 mm) were packed with pellicular silica (40–63 μm), both obtained from Alltech (Carnforth, UK). The chiral column, a Chiralpak AD (amylose tris(3,5-dimethylphenylcarbamate)) column (250×4.6 mm, 10 μm), used with a matching guard column (50×4.6 mm, 10 μm), was supplied by HPLC Technology Ltd. (Macclesfield, UK).

2.3. Instrumentation

High-performance liquid chromatography (HPLC) was performed using a LDC Constametric 3000 pump, a LDC Spectromonitor 3100 UV detector and a LDC CI 4000 computing integrator (Stone, UK). Samples were injected using a LKB 2157 autosampler (Pharmacia, Milton Keynes, UK) or a Perkin-Elmer ISIS 100 autosampler (Beaconsfield, UK). All glassware used in the extraction procedures was silanized prior to use.

2.4. Solvent extraction

2.4.1. Determination of unconjugated metabolites

To aliquots of urine (0.5 ml) was added 4-chlorophenoxyacetic acid (25 μg; 50 μl of a 0.5 mg/ml solution in acetonitrile) as internal standard. The samples were then acidified by adding hydrochloric acid (1.0 *M*; 100 μl) and buffered to pH 3.8 with 1.5 ml of phosphate buffer (pH 3.8, 1.0 *M*). Dichloromethane–ethyl acetate (14:1, v/v; 5 ml) was added and the extraction tubes were then tightly capped and mixed on a test-tube rocker for 20 min. Phase separation was achieved by centrifugation for 5 min at 1000 *g*. The lower organic layer was then transferred into a clean glass tube and evaporated to dryness under a gentle stream of nitrogen at 40°C on a dry heating block. The residue was reconstituted in 150 μl mobile phase and 50 μl was injected into the achiral HPLC system.

2.4.2. Determination of total (free plus conjugated) metabolite concentrations

To aliquots of urine (0.1 ml) was added 4-chlorophenoxyacetic acid (25 μg; 50 μl of a 0.5 mg/ml solution in acetonitrile) as internal standard followed by NaOH (1.0 *M*; 20 μl) for hydrolysis of the acyl glucuronic acid conjugates. The hydrolysis reaction was left to proceed for 2 h at room temperature, after this time HCl (1.0 *M*; 40 μl), phosphate buffer (pH 3.8, 1.0 *M*; 200 μl) and dichloromethane–ethyl acetate (14:1, v/v; 1 ml) as extraction solvent were added. The tubes were mixed and extraction carried out as before. All analysis of urine samples was carried out in duplicate.

2.5. Chromatographic conditions

The achiral phase used was a Partisil 5 silica column protected by a guard column filled with pellicular silica. The mobile phase consisted of hexane–ethanol (98.2:1.8, v/v) containing trifluoroacetic acid (0.05%, v/v) as modifier, at a flow-rate of 2 ml/min at ambient temperature. The UV detector was set at 220 nm and the detector output was set at 0.02 a.u.f.s.

2.6. Validation of the normal-phase HPLC procedure

A series of calibration standards containing 10, 20, 40, 80, 160 and 320 μg/ml of each metabolite were prepared in drug-free urine. On each day of analysis, 0.5 ml of these standard solutions were analysed together with volunteer samples. Calibration curves

were constructed by plotting peak area ratios (metabolite/internal standard) against concentration of the metabolite concerned and subjecting this data to linear regression analysis. The concentrations in volunteer urine and prepared quality control samples were determined by interpolation from the regression equations of the respective calibration curves.

The accuracy and within day variation of the assay was assessed by analysing six prepared quality control samples containing 10, 80 and 320 μg/ml of both metabolites. The precision and accuracy of the assay was determined for each metabolite by calculation of the percentage coefficient of variation, C.V.% (S.D./mean×100%) and the mean percentage difference (mean measured concentration−actual concentration/actual concentration×100%). The recovery of the extraction procedure was determined by comparing the peak areas obtained with extracted samples to those of direct injections of standard solutions of the same concentration ($n=6$).

The between day variation of the assay was determined by analysing the above samples on 6 consecutive days. The coefficient of variation and accuracy were calculated as before.

2.7. Fraction collection

The column eluate from the achiral phase containing carboxy (t_R, 12.6 min; fraction collected between 12.2 and 13.0 min) and hydroxyibuprofen (t_R, 15.8 min; fraction collected between 15.3 and 16.3 min) were separately collected from the detector outlet into silanized tubes. The eluate was gently evaporated under nitrogen gas at 40°C in a dry block heater and the residue reconstituted in 100 μl mobile phase and subjected to chiral-phase analysis.

2.8. Chiral-phase analysis of carboxyibuprofen and hydroxyibuprofen in urine

Standard metabolite solutions dissolved in mobile phase, or eluate collected from the normal-phase analysis redissolved in the mobile phase used for chiral chromatography, were injected in 50 μl aliquots onto the HPLC. The mobile phase was hexane–ethanol (92:8, v/v) with trifluoroacetic acid (0.05%, v/v) as modifier, run at a flow-rate of 1 ml/min. The detection wavelength was set at 220 nm. The column used was a Chiralpak AD CSP (250×4.6 mm, 10 μm) connected to a guard column containing similar material (50×4.6 mm, 10 μm).

The enantiomeric composition of hydroxyibuprofen was determined as follows:

$$\text{Enantiomeric composition} = \frac{\text{Peak area of enantiomer}}{\text{Total peak area of both enantiomers}}$$

Individual concentrations of each enantiomer were calculated by multiplying their enantiomeric composition by the corresponding metabolite concentrations obtained from the normal-phase analysis. For carboxyibuprofen, the same approach was used, the total peak area being for all four stereoisomers.

2.9. Validation of the chiral assay procedure

In order to determine whether the chiral HPLC method could produce accurate stereochemical compositions over a wide range of concentrations, the HPLC eluate from the validation experiments described above were collected and subjected to chiral-phase analysis. The stereochemical composition of both metabolites and the coefficient of variation and mean percentage difference were calculated as described above.

2.10. Application of the analytical method

Three healthy volunteers (two males aged 36 and 22 years, weight 59 and 77 kg, respectively, and one female, aged 29 years, weight 68 kg) were administered a single oral dose of racemic ibuprofen, as a 400 mg tablet, following an overnight fast. Sequential urine samples were collected at the following time intervals post drug administration: 0–2, 2–4, 4–6, 6–8, 8–10 and 10–24 h and the individual urine volumes recorded. A pooled 24 h urine sample was prepared from the individual samples and a 50 ml aliquot retained for analysis. All urine samples were stored frozen at −20°C until required for analysis.

The enantiomeric composition of ibuprofen in urine, both free and conjugated with glucuronic acid, was determined using the indirect approach to enantiomeric analysis. Following extraction, the drug was derivatized with (*R*)-1-(naphthen-1-yl)-ethylamine and the enantiomeric composition

determined by reversed-phase HPLC [32].

3. Results and discussion

When CSPs are used in bioanalysis, separation problems are frequently encountered due to the restricted choice of solvent conditions available. Also, the presence of co-extracted contaminants may cause problems in analyte retention, resolution and column stability [25]. An additional problem is the possible co-elution of the enantiomers of the analyte of interest with those of a structurally similar metabolite. Some of these problems associated with CSPs can be overcome by using coupled column chromatography, where drug concentrations are determined on an achiral phase and the appropriate fraction of the eluate transferred to a CSP via a switching valve, for the determination of enantiomeric composition [26]. In the present investigation this approach seemed appropriate due to the number of stereoisomers of carboxyibuprofen, together with the requirement to determine the stereochemical composition of both metabolites. However, a sequential approach was adopted to obtain a high throughput of samples and to increase assay sensitivity in the chiral separation stage by concentrating the collected eluate from the achiral phase before introduction onto the CSP.

Previously reported HPLC methods for the analysis of hydroxy- and carboxyibuprofen have utilized reversed-phase systems [13–15] and in some cases gradient elution [12,16]. The choice of normal-phase over reversed-phase chromatography for the achiral stage in the present method was based on the ease of sample manipulation, i.e. solvent evaporation following the collection of the required eluate fractions, together with mobile-phase compatibility. Had reversed-phase chromatography been used, the aqueous solvents would have to be freeze-dried, or the analytes extracted, which could result in a reduction in metabolite recovered, with subsequent sensitivity problems with the chiral analysis. In addition, salt residues from the buffers used in reversed-phase analysis may affect the stability or efficiency of the CSP. Under the chromatographic conditions used, the retention times of the internal standard, carboxyibuprofen and hydroxyibuprofen were 4.9, 12.6 and 15.8 min, respectively (Fig. 2).

Examination of potential extraction solvents indicated that dichloromethane gave the chromatographically cleanest extracts but analyte recovery was only

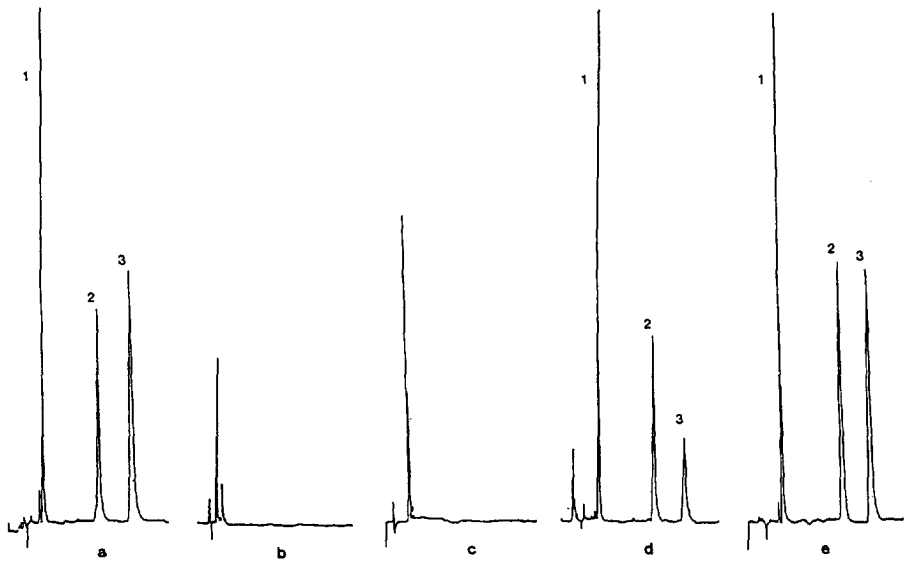

Fig. 2. Normal-phase chromatograms of: (a) a standard solution containing 100 μg/ml of *p*-chlorophenoxyacetic acid (1, 4.9 min) and 180 μg/ml each of carboxyibuprofen (2, 12.6 min) and hydroxyibuprofen (3, 15.8 min); (b) blank urine extract; (c) alkali-treated blank urine extract; (d,e) extracts of a urine sample from a volunteer obtained 2–4 h following the oral administration of racemic ibuprofen (400 mg), pre- (d) and post- (e) treatment of the urine with alkali. For chromatographic conditions see Section 2.

Table 1
Within-day and between-day analytical variation and accuracy of carboxyibuprofen in prepared quality control urine samples (mean±S.D., $n=6$)

Concentration (μg/ml)	Concentration determined (μg/ml)	Coefficient of variation (C.V.%)	Mean percent difference
Within-day			
10	10.22±0.33	3.2	2.16
80	79.94±0.46	0.6	−0.08
320	322.9±11.82	3.7	0.93
Between-day			
10	9.66±0.68	7.0	−3.40
80	82.68±6.67	8.1	3.36
320	325.7±30.9	9.5	1.79

40% with a single 5 ml extraction. This observation is consistent with that of Rudy et al. [20] where a 2×7 ml extraction procedure was used. Mixtures of dichloromethane and ethyl acetate were then examined and it was found that a proportion of 14:1 (v/v) gave good recoveries with low background noise (Fig. 2). The extraction recovery was determined at three concentrations for both analytes and the following results were obtained (concentration, mean±S.D., $n=6$): Carboxyibuprofen: 10 μg/ml, 94.2±3.0%; 80 μg/ml, 93.1±0.5%; 320 μg/ml, 90.5±3.3%; Hydroxyibuprofen: 10 μg/ml, 96.6±6.6%; 80 μg/ml, 97.1±2.7%; 320 μg/ml, 93.2±4.9%. No interfering peaks were observed in the chromatograms at the retention times of either metabolite or the internal standard owing to endogenous constituents of urine either before or after alkali treatment to hydrolyse the ester glucuronide conjugates (Fig. 2). The use of alkaline rather than enzymatic hydrolysis of acyl glucuronides circumvents analytical problems associated with the intramolecular acyl migration of the carboxylic acid moiety to yield β-glucuronidase-resistant glucuronic acid esters [27,28].

Linear regression analysis of the calibration curves routinely gave regression coefficients better than 0.997. The limit of quantitation for the procedure was set at 10 μg/ml for both metabolites in urine. However, a lower limit of detection can be easily achieved by analysing a larger volume of urine or by injecting a larger proportion of the extract on-column. In the present study, this was not deemed necessary as the quantity of both analytes in urine resulting from a typical oral dose of ibuprofen is relatively high. The analytical procedure showed acceptable precision, accuracy and between day variability for both metabolites at the three concentration levels examined (Tables 1 and 2).

3.1. Chiral resolution of carboxyibuprofen and hydroxyibuprofen enantiomers

The chromatographic conditions used for the stereochemical analysis of carboxyibuprofen were

Table 2
Within-day and between-day analytical variation and accuracy of hydroxyibuprofen in prepared quality control urine samples (mean±S.D., $n=6$)

Concentration (μg/ml)	Concentration determined (μg/ml)	Coefficient of variation (C.V.%)	Mean percent difference
Within-day			
10	10.24±0.70	6.8	2.36
80	79.85±2.23	2.8	−0.18
320	320.7±16.8	5.2	0.21
Between-day			
10	9.91±0.70	7.0	−0.92
80	80.37±4.32	5.4	0.46
320	314.5±30.7	9.8	−1.73

similar to those reported previously [24]. Under the conditions used, the elution order of the diastereoisomers is 2′*S*,2*R*-, 2′*R*,2*R*- 2′*R*,2*S*- and 2′*S*,2*S*- with retention times of 11.0, 12.1, 16.9 and 20.1 min, respectively (Fig. 3). The separation (α) and resolution (R_s) factors for the four peaks were 1.2, 1.5 and 1.3, and 1.3, 3.3 and 2.4, respectively, indicating good baseline separation, when compared to work published previously [7,20,23]. The same mobile-phase conditions were also used for the analysis of hydroxyibuprofen. The enantiomeric elution order was determined to be (*R*)- before (*S*)-hydroxyibuprofen, with retention times of 13.9 and 16.1 min, respectively. The separation was baseline with separation and resolution factors of 1.25 and 1.6, respectively (Fig. 4).

The individual collection of both metabolites following normal-phase chromatography although tedious, yielded chiral-phase chromatograms which were generally free from interferences. Had the urinary extracts been injected directly onto the CSP, under the conditions employed, co-elution of (*R*)-hydroxyibuprofen with (2′*R*,2*R*)-carboxyibuprofen and (*S*)-hydroxyibuprofen with (2′*R*,2*S*)-carboxyibuprofen would have occurred. Although separation and resolution of all six analytes was achieved by using a mobile phase consisting of hexane–ethanol–methanol (95:3.5:1.5, v/v) containing trifluoroacetic acid (0.05%, v/v) as modifier, the resolution was poorer, the overall run time was relatively long, ca. 1 h and an increase in band broadening was observed (Fig. 5). By separately analysing the carboxy- and hydroxyibuprofen fractions, better resolution was achieved and the overall analysis times were approximately the same.

Analysis of standard solutions of 'racemic' carboxyibuprofen yielded peak areas with stereoisomer fractions of 0.25 each. Similarly, the enantiomeric composition of hydroxyibuprofen following analysis of the racemate was 1:1. The chiral analysis method was validated by assaying the material in the eluate collected from the normal-phase validation experiments. These validation experiments are essential to determine whether the chiral assay was able to reproduce the expected enantiomeric compositions throughout the entire analytical procedure and over the range of analyte concentrations. The precision and accuracy values of the calculated stereochemical

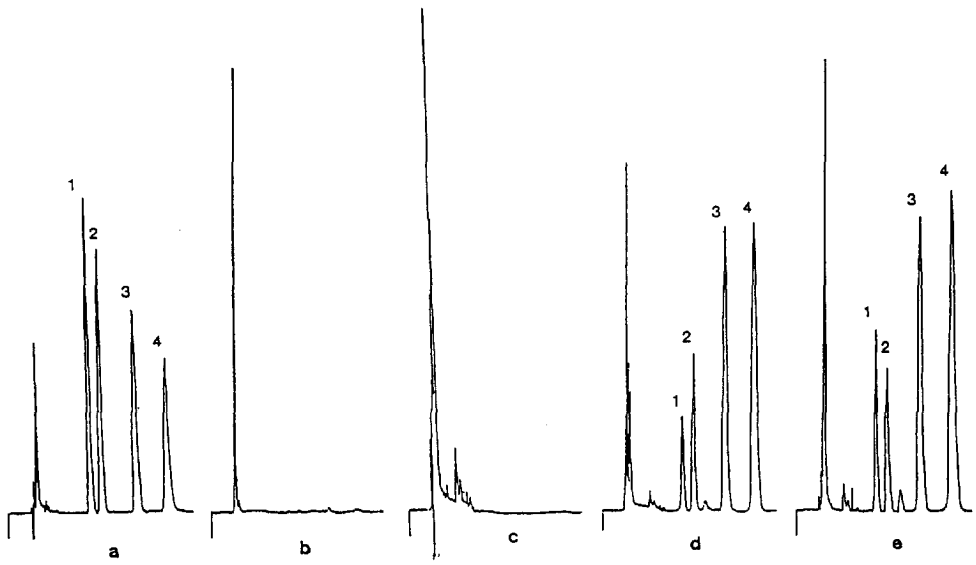

Fig. 3. Chiral-phase chromatograms of (a) a standard solution of 'racemic' carboxyibuprofen (80 μg/ml). Retention times of diastereoisomers: (1) (2′*S*,2*R*)-, 11.0 min; (2) (2′*R*,2*R*)-, 12.1 min; (3) (2′*R*,2*S*)-, 16.9 min; (4) (2′*S*,2*S*)-, 20.1 min. (b) Eluate from blank urine analysed by normal-phase HPLC; (c) eluate from alkali-treated blank urine analysed by normal-phase HPLC. (d,e) Eluates from the normal-phase HPLC analysis of a urine sample obtained from a volunteer 2–4 h following the oral administration of racemic ibuprofen (400 mg), pre- (d) and post- (e) treatment of the urine with alkali. For chromatographic conditions see Section 2.

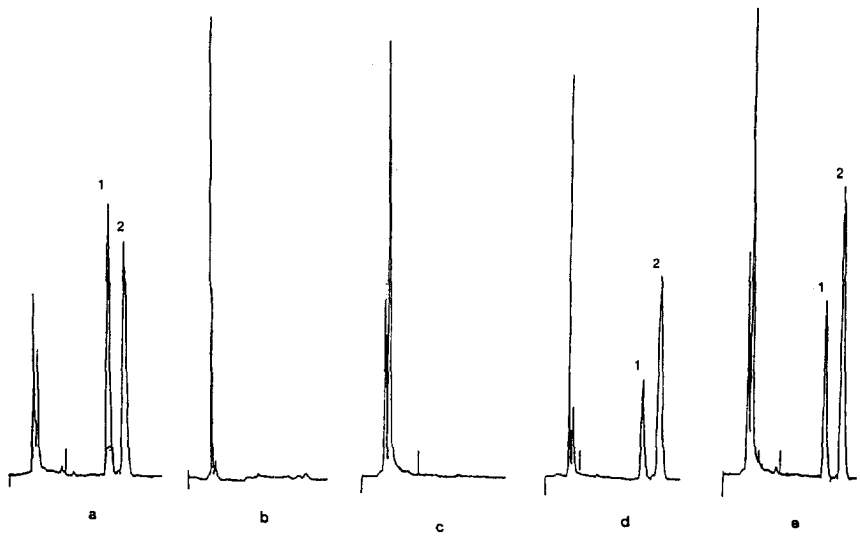

Fig. 4. Chiral-phase chromatograms of (a) a standard solution of racemic hydroxyibuprofen, 80 μg/ml. Retention times: (1) (*R*)-hydroxyibuprofen, 13.9 min; (2) (*S*)-hydroxyibuprofen, 16.1 min. (b) Eluate from blank urine analysed by normal-phase HPLC. (c) Eluate from alkali-treated blank urine analysed by normal-phase HPLC. (d,e) Eluates from the normal-phase HPLC analysis of a urine sample obtained from a volunteer 2–4 h following the oral administration of racemic ibuprofen (400 mg), pre- (d) and post- (e) treatment of the urine with alkali. For chromatographic conditions see Section 2.

compositions for both metabolites are presented in Tables 3 and 4. The results indicate little variation from the expected stereochemical compositions over the wide range of concentrations examined, even at the lowest concentration used (10 μg/ml), the chiral-phase procedure was sufficiently sensitive to determine the stereochemical composition of the two

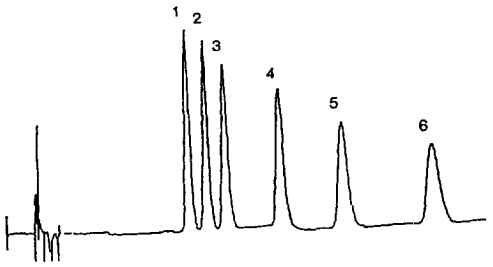

Fig. 5. Chiral-phase chromatogram of a mixture of carboxyibuprofen and hydroxyibuprofen analysed with a mobile-phase composition of hexane–methanol–ethanol (95:3.5:1.5, v/v) containing trifluoroacetic acid (0.05%, v/v), flow-rate 1.0 ml/min. Retention times: (1) (2′*S*,2*R*)-carboxyibuprofen, 20.9 min; (2) (*R*)-hydroxyibuprofen, 23.0 min; (3) (2′*R*,2*R*)-carboxyibuprofen, 25.4 min; (4) (*S*)-hydroxyibuprofen, 31.6 min; (5) (2′*R*,2*S*)-carboxyibuprofen, 38.9 min; (6) (2′*S*,2*S*)-carboxyibuprofen, 49.4 min.

metabolites in the eluate from the normal-phase system in a reproducible manner.

3.2. Application of the analytical method in metabolic studies

The method was used to analyse urine samples collected at 2 h intervals from 0 to 10 h followed by a pooled urine sample from 10 to 24 h from three healthy volunteers following the oral administration of 400 mg of racemic ibuprofen. The total recovery of ibuprofen and both metabolites, both free and conjugated with glucuronic acid is presented in Table 5. Of the administered dose some 22.7 and 39.7% was excreted as the hydroxy and carboxy metabolites, respectively. These results are in good agreement with previously published data [7,11,29]. The urinary recovery of (*S*)-hydroxyibuprofen showed marked stereoselectivity compared to (*R*)-hydroxyibuprofen ($S/R=5.9$) and examination of the free versus conjugated recovery indicated preferential conjugation of the *S*-enantiomer (ratio conjugated/free: $S=3.18$; $R=0.61$). Although the enrichment of the urine in (*S*)-hydroxyibuprofen could be due to

Table 3
Within-day and between-day variation and accuracy of the stereochemical composition of racemic carboxyibuprofen determined by the analysis of the eluate from the achiral normal-phase analysis of prepared quality control urine samples ($n=6$)

Concentration (μg/ml)	Isomer	Stereochemical composition (%) (mean±S.D.)	Coefficient of variation (C.V.%)	Mean % difference
Within-day				
10	2′*S*,2*R*	25.05±1.24	4.99	0.21
	2′*R*,2*R*	25.10±1.79	7.13	0.41
	2′*R*,2*S*	25.01±0.51	2.04	0.04
	2′*S*,2*S*	24.84±2.42	9.75	−0.66
80	2′*S*,2*R*	24.97±0.71	2.87	−0.13
	2′*R*,2*R*	25.05±1.52	6.10	0.22
	2′*R*,2*S*	24.99±0.97	3.87	−0.05
	2′*S*,2*S*	24.99±1.17	4.68	−0.03
320	2′*S*,2*R*	25.04±1.34	5.34	0.14
	2′*R*,2*R*	25.01±1.04	4.16	0.02
	2′*R*,2*S*	24.99±1.20	4.81	−0.03
	2′*S*,2*S*	24.97±0.59	2.36	−0.13
Between-day				
10	2′*S*,2*R*	24.99±1.05	4.21	−0.06
	2′*R*,2*R*	25.07±1.75	6.98	0.26
	2′*R*,2*S*	24.99±1.14	4.55	−0.04
	2′*S*,2*S*	24.96±0.47	1.88	−0.17
80	2′*S*,2*R*	24.98±0.35	1.41	−0.07
	2′*R*,2*R*	25.02±1.02	4.06	0.09
	2′*R*,2*S*	24.98±0.33	1.31	−0.08
	2′*S*,2*S*	25.02±0.47	1.87	0.06
320	2′*S*,2*R*	25.06±1.66	6.63	0.22
	2′*R*,2*R*	24.97±0.73	2.94	−0.13
	2′*R*,2*S*	25.02±1.66	6.65	0.06
	2′*S*,2*S*	24.96±0.88	3.52	−0.15

Table 4
Within-day and between-day variation and accuracy of the stereochemical composition of racemic hydroxyibuprofen determined by the analysis of the eluate from the achiral normal-phase analysis of prepared quality control urine samples ($n=6$)

Concentration (μg/ml)	Isomer	Enantiomeric composition (%) (mean±S.D.)	Coefficient of variation (C.V.%)	Mean % difference
Within-day				
10	*R*	50.00±0.36	0.71	0.00
	S	50.00±0.36	0.71	0.00
80	*R*	50.02±1.61	3.21	0.04
	S	49.98±1.60	3.21	−0.04
320	*R*	50.00±0.98	1.96	0.01
	S	50.00±0.98	1.96	0.00
Between-day				
10	*R*	50.05±2.50	5.00	0.09
	S	49.95±2.50	5.01	−0.09
80	*R*	50.05±1.39	2.77	0.10
	S	49.95±1.38	2.77	−0.10
320	*R*	50.12±1.68	3.35	0.25
	S	49.88±1.68	3.37	−0.25

Table 5
Urinary excretion (0–24 h) of ibuprofen and hydroxyibuprofen enantiomers and carboxyibuprofen stereoisomers following the administration of the racemic drug (400 mg) to three healthy volunteers (data expressed as a percentage of the administered dose)

	Mean	S.D.
(*R*)-Ibuprofen		
free:	0.06	0.03
conjugated:	1.20	0.47
total	1.26	0.50
(*S*)-Ibuprofen		
free:	0.39	0.11
conjugated:	8.15	1.39
total	8.54	1.47
(*R*)-Hydroxyibuprofen		
free:	2.05	0.67
conjugated:	1.25	0.17
total	3.30	0.82
(*S*)-Hydroxyibuprofen		
free:	4.64	1.89
conjugated:	14.78	1.63
total	19.42	1.63
(2′*S*,2*R*)-Carboxyibuprofen		
free:	1.47	0.17
conjugated:	1.23	0.29
total	2.70	0.19
(2′*R*,2*R*)-Carboxyibuprofen		
free:	2.02	0.59
conjugated:	2.81	0.54
total	4.83	0.94
(2′*R*,2*S*)-Carboxyibuprofen		
free:	6.53	1.59
conjugated:	7.61	0.51
total	14.14	2.05
(2′*S*,2*S*)-Carboxyibuprofen		
free:	8.64	2.60
conjugated:	9.43	2.05
total	18.07	4.62
Total recovery	72.26	9.25

the metabolic chiral inversion of (*R*)- to (*S*)-ibuprofen, the marked difference in the amounts of the *R*- and *S*-enantiomers of hydroxyibuprofen indicates that stereoselective oxidation of (*S*)-ibuprofen is the more likely cause as the fraction undergoing chiral inversion is reported to be between 0.52 and 0.74 [29–31]. Thus, based on urinary recovery, (*S*)-ibuprofen shows substrate stereoselectivity with respect to formation of the alcohol and the metabolite appears to show substrate selectivity for conjugation. Examination of the urinary excretion for the sum of the diastereoisomers of carboxyibuprofen arising from oxidation of either enantiomer of ibuprofen indicates substrate stereoselectivity for (*S*)-ibuprofen ($S/R=4.3$). Examination of the corresponding data for the individual stereoisomers indicates product stereoselectivity for the formation of a particular diastereoisomer. Thus, oxidation of ibuprofen to yield carboxyibuprofen shows substrate–product stereoselectivity, with the predominantly formed diastereoisomer having the same configuration at the metabolically introduced centre as that in the substrate in both cases, i.e. oxidation of (*R*)-ibuprofen preferentially yields (2′*R*,2*R*)-carboxyibuprofen, whereas (*S*)-ibuprofen preferentially yields (2′*S*,2*S*)-carboxyibuprofen.

The conjugation of carboxyibuprofen also appears to show some stereoselectivity, for example the mean ratios of conjugate:free for the four stereoisomers are: 0.8, 1.4, 1.2, and 1.1 for the 2′*S*,2*R*, 2′*R*,2*R*, 2′*R*,2*S*, and 2′*S*,2*S*-stereoisomers, respectively. Thus, the 2′*S*,2*R*- stereoisomer appears to be a poorer substrate for conjugation than the other three carboxyibuprofen stereoisomers. The significance of this data is, however, limited as at present the regioselectivity, i.e. the position of conjugation is unknown.

4. Conclusion

In summary, a reliable normal-phase HPLC assay has been developed and validated for the determination of carboxyibuprofen and hydroxyibuprofen in urine. Extension of the methodology by the addition of a sequential chiral-phase analysis step, using a Chiralpak AD CSP, has resulted in the development of an assay suitable for the determination of the stereochemical composition of both metabolites in urine. The methodology described above is the first reported method for the determination of the stereochemical composition of the two major metabolites of ibuprofen in urine involving sequential achiral–chiral chromatography and the first method in which the four stereoisomers of carboxyibuprofen have been unequivocally quantified following administration of the racemic drug. Previously reported meth-

ods for the analysis of carboxyibuprofen have all resulted in co-elution of two of the stereoisomers, or their derivatives [7,18,19,23], or involved long retention times with chromatographic run times of greater than 1 h [20]. The application of this methodology to an examination of the stereochemistry of ibuprofen metabolism in man is currently in progress and will be reported elsewhere.

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