Journal of Chromatography, 494 (1989) 173-182
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4860

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE ENANTIOMERS OF FLURBIPROFEN AND ITS METABOLITES IN PLASMA AND URINE

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(First received February 28th, 1989, revised manuscript received May 10th, 1989)

SUMMARY

Reversed-phase high-performance liquid chromatographic methods have been developed to quantitate the *R*- and *S*-enantiomers of flurbiprofen and its major metabolites, 4′-hydroxyflurbiprofen, 3′-hydroxy-4′-methoxyflurbiprofen, and 3′,4′-dihydroxyflurbiprofen The compounds are extracted from plasma or urine and derivatized with *S*-(α)-methylbenzylamine to form diastereomeric amides which are readily separated on a C_{18} column Fluorescence detection resulted in detection limits that readily allowed us to characterize the disposition of *R*- and *S*-flurbiprofen and its major metabolites in man following therapeutic doses

INTRODUCTION

Flurbiprofen is an arylpropionic acid non-steroidal anti-inflammatory drug used in the treatment of arthritis and mild to moderate pain [1,2] In humans, flurbiprofen is eliminated primarily via metabolism either by conjugation to glucuronide or sulfate conjugates or by oxidation to 4′-hydroxyflurbiprofen (4′-OHF), 3′-hydroxy-4′-methoxyflurbiprofen (3′-OH-4′-OCH_3), and 3′,4′-dihydroxyflurbiprofen (3′,4′-OHF) (Fig 1) The oxidative metabolites also undergo conjugation before appearing in urine [3–5]

Flurbiprofen contains an asymmetric carbon atom and is administered as a racemate despite the fact that the therapeutic activity is believed to reside predominantly in the *S*-enantiomer [6] All of the identified metabolites retain the asymmetric center and therefore may exist in the *R*- or *S*-configura-

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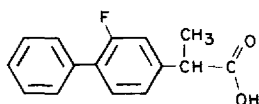

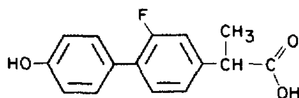

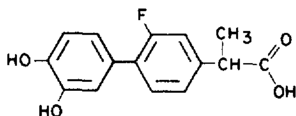

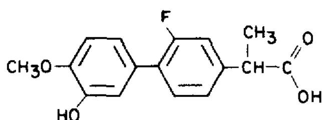

Fig 1 Structures of flurbiprofen and its major metabolites

tion It is known that the disposition of enantiomers of chiral drugs and metabolites may differ, stereoselective distribution, metabolism, elimination, or protein binding may occur Dispositional studies therefore need to employ methods that distinguish between enantiomers of both parent drug and metabolites Previously reported methods for assaying plasma or urine for flurbiprofen and metabolites have quantified total parent drug [7–13], total metabolites [5,14], or the enantiomers of parent drug only [15].

Maître et al [16] described the use of (α)-methylbenzylamine to form diastereomeric amide derivatives of arylpropionic acids which were separated by normal-phase high-performance liquid chromatography (HPLC) The carboxyl group that participates in amide formation is retained by the principle oxidative metabolites of flurbiprofen, therefore, we extended the above technique to allow quantitation of the enantiomers of the metabolites, as well as parent compound, in biological fluids. Resolution of the diastereomeric product was readily achieved by reversed-phase HPLC.

EXPERIMENTAL

Materials

R-, *S*-, and racemic flurbiprofen were provided by Upjohn (Kalamazoo, MI, U S A) Racemic 4′-OHF and 3′-OH-4′-OCH_3 were obtained from Boots (PLC, Nottingham, U.K.), and 3′,4′-OHF was a gift from Dr J G Wagner (University of Michigan, Ann Arbor, MI, U S A) The 1,1′-carbonyldiimidazole and the *S*-(α)-methylbenzylamine were purchased from Aldrich (Milwaukee, WI, U S A.). The latter has been shown to be essentially free of optical impurity [17] The internal standard, racemic ibuprofen, was purchased from Sigma (St Louis, MO, U S.A.) and naproxen was obtained from Syntex Labs (Palo Alto, CA, U S.A.) Chemicals and organic solvents were purchased from Fisher Scientific (Fairlawn, NJ, U S A.)

Stock solutions of flurbiprofen, metabolites, and internal standard were prepared in methanol at either 1 or 0 1 mg/ml and diluted with water to obtain the working solutions at a concentration of 10 ng/μl A solution of 65 mg/ml 1,1′-carbonyldiimidazole was prepared in chloroform daily

Apparatus and chromatographic conditions

The HPLC system consisted of a Beckman Model 114M pump (Beckman, San Ramon, CA, U.S A), a Spectra Physics SP8780 autosampler (Spectra Physics, San Jose, CA, U.S A.) fitted with a 200-μl sample loop, and either a Kratos 783 UV detector (Kratos Analytical, Ramsey, NJ, U S A) or an Applied Biosystems 980 programmable fluorescence detector (Applied Biosystems, Ramsey, NJ, U S.A) connected to a Beckman Ultrasphere ODS column (25 cm×4 6 mm I.D , 5-μm particles, Beckman) protected by a Brownlee RP18 guard column (Brownlee Labs., Santa Clara, CA, U S A) All analyses were performed at room temperature The mobile phase varied with the compound being analyzed For measurement of *R*- and *S*-flurbiprofen in plasma a mobile phase of acetonitrile–water (62 38, v/v) was employed but in urine samples a mobile phase of acetonitrile–0 05 *M* acetic acid (55 45, v/v) allowed simultaneous analysis of flurbiprofen and 3′,4′-OHF; both were at a flow-rate of 1 ml/min For measurement of the enantiomers of 4′-OHF and 3′-OH-4′-OCH_3 in both plasma and urine, the mobile phase was acetronitrile–tetrahydrofuran–0 05 *M* acetic acid (43 2 55, v/v) at a 1 1 ml/min flow-rate Ultraviolet detection at 245 nm was used for monitoring flurbiprofen in plasma, but fluorescence detection was used for urine and metabolite analyses When monitoring for flurbiprofen and 3′,4′-OHF simultaneously in urine the excitation wavelength was 200 nm and the emission cutoff filter was 320 nm, but for the other two metabolites the excitation wavelength was 260 nm with a 320-nm emission cutoff filter

The elution order of flurbiprofen enantiomers was assessed by derivatizing the individual enantiomers. In the case of the metabolites, the elution order

was determined by analyzing urine from a rat dosed intravenously with 2 1 mg/kg *S*-flurbiprofen The *S*-enantiomer retains its configuration and therefore the urinary metabolites are also of the *S*-configuration [6]

Flurbiprofen in plasma

Aliquots of plasma (usually 500 μl) were pipetted into glass extraction tubes with screw tops and the volume was adjusted to 1 ml with water After the addition of the internal standard, ibuprofen (15 μg per 100 μl), 2 ml of 10% trichloroacetic acid were added to precipitate the proteins and acidify the plasma Standard curve samples at concentrations ranging from 25 to 500 ng/ml of the individual enantiomers were prepared similarly in drug-free human plasma For increased recovery the compounds were extracted twice using 5 ml hexane and gentle mixing for 15 min Between extractions, the samples were centrifuged for 5–10 min at 800 *g* The combined hexane extracts were evaporated under a gentle stream of nitrogen at a temperature of 37–40°C

The sample residues were reconstituted with 300 μl chloroform and then 200 μl of the 1,1′-carbonyldiimidazole solution were added After a 5–10 min incubation at room temperature, 10 μl glacial acetic acid were added, then vortexed briefly The compounds were allowed to react an additional 5–10 min before 50 μl *S*-(α)-methylbenzylamine were added to the solution and mixed briefly The derivatization reaction proceeded at room temperature for 30 min and was stopped by the addition of 3 ml of 0 5 *M* ammonium hydroxide Longer reaction times did not increase the yield of derivative as also noted by others [16] The diastereomeric amide derivatives were extracted into 5 ml hexane by gentle mixing for 15 min The hexane was removed and washed with 3 ml of 1 *M* hydrochloric acid followed by another 3 ml of 0 5 *M* ammonium hydroxide and an additional 3 ml of 1 *M* hydrochloric acid Each time the solutions were mixed for 15 min After the final wash, the hexane was evaporated under a gentle stream of nitrogen at 37°C Several washes were needed to completely remove excess reagents and contaminants that interfered chromatographically The residue was dissolved in 150 μl mobile phase and 25 μl were injected onto the column

Flurbiprofen and metabolites in urine

Urine samples were analyzed for flurbiprofen and its metabolites both before and after hydrolysis Acid hydrolysis was routinely employed and involved the addition of 1 ml of 6 *M* hydrochloric acid to 1 ml of urine followed by incubation at 90°C for 30 min Spiked urine samples indicted that there was no loss of flurbiprofen or metabolites during this procedure Preliminary studies indicated that hydrolysis with acid, alkali, and β-glucuronidase all gave chromatograms free of interferences and linear calibration lines, but β-glucuronidase failed to completely hydrolyse flurbiprofen conjugates Acid hydrolysis was

chosen over base to facilitate the extraction procedure and to ensure that both ester and ether conjugates were hydrolysed [18]

Due to their different polarities, flurbiprofen and its metabolites could not be conveniently analyzed in a single isocratic system Following hydrolysis samples were cooled to room temperature and the internal standard was added The internal standard for flurbiprofen and 3′,4′-OHF was ibuprofen (15 μg per 100 μl) and for 4′-OHF and 3′-OH-4′-OCH_3 it was naproxen (400 ng per 40 μl) Standard curve samples were prepared in drug-free urine and were treated similarly

The compounds of interest were extracted into 5 ml dichloroethane by mixing for 15 min The dichloroethane was evaporated under a stream of nitrogen at 37–40°C The compounds were derivatized with *S*-(α)-methylbenzylamine as described previously for plasma except 0 25 instead of 0 5 *M* ammonium hydroxide was used After derivatization, the diastereomeric amides were extracted into 5 ml of dichloroethane which was washed with 3 ml of 0 25 *M* ammonium hydroxide and twice with 3 ml of 1 *M* hydrochloric acid The dichloroethane was evaporated under a stream of nitrogen at 37–40°C The residue was reconstituted with 150 μl acetonitrile–water (45 55, v/v) and 20–30 μl were injected

Metabolites in plasma

Plasma samples could be analyzed for conjugates of flurbiprofen, by comparing the concentration of flurbiprofen before and after hydrolysis The procedure for hydrolysis and quantitation of flurbiprofen conjugates in plasma was identical to that for urine but hexane was employed in place of dichloroethane In addition, plasma could also be assayed for the two major metabolites of flurbiprofen, 4′-OHF and 3′-OH-4′-OCH_3 These metabolites were extracted into dichloroethane from plasma after acidification with 1 ml of 1 *M* hydrochloric acid The derivatization and chromatographic procedure was the same as for urine

Data analysis

A five-point standard curve prepared in the appropriate biological fluid was analyzed with each set of samples Some sample concentrations exceeded the range of a standard curve and were therefore diluted appropriately and reassayed Quantitation was based on least-squares linear regression analysis of concentration versus peak-height ratio. One of the two ibuprofen internal standard peaks was chosen and used throughout a set of calculations The assay accuracy and precision were assessed by the relative error and the coefficient of variation (C V) The relative error, expressed as percentage, was calculated by dividing the mean value by the actual value

RESULTS AND DISCUSSION

Derivatization of arylpropionic acids, such as flurbiprofen, with S-(α)-methylbenzylamine to form diastereomeric amides has been shown to be useful in the separation of these compounds on achiral columns [16] Commercially available S-(α)-methylbenzylamine has been shown to be an optically pure derivatizing reagent [17], and we have successfully extended this approach to the separation of the enantiomers of the metabolites, in addition to flurbiprofen, in both urine and plasma

Fig 2 shows a chromatogram of flurbiprofen extracted from spiked human plasma The peak that corresponds to the S-enantiomer (retention time 12 0 min) elutes before that of the R-enantiomer for both flurbiprofen and ibuprofen. The second ibuprofen peak was used routinely as the internal standard, although either peak yielded acceptable results No extraneous peaks in the area of the flurbiprofen enantiomers or the internal standard were seen in blank plasma extracts (Fig. 2A) Although levels as low as 10 ng/ml could be detected, the lowest level routinely quantified was 25 ng/ml of each enantiomer Standard curves prepared in diluted plasma over the range 25–500 ng/ml of each enantiomer were linear (r^2>0 99) The intra- and inter-day C V was less than 10% at concentrations of 25, 250, and 500 ng of each enantiomer, for example, at 250 ng, the C V was 4 4 and 4 7% (n=15) for S- and R-flurbiprofen, respectively The relative error was also less than 10% (4 3 and 4 0%, n=15, at 250 ng S- and R-flurbiprofen, respectively)

Since the majority of flurbiprofen and its metabolites are found in urine as either glucuronide or sulfate conjugates [3–5], urine samples were hydrolyzed with acid and heat before analysis When urine was assayed for flurbiprofen,

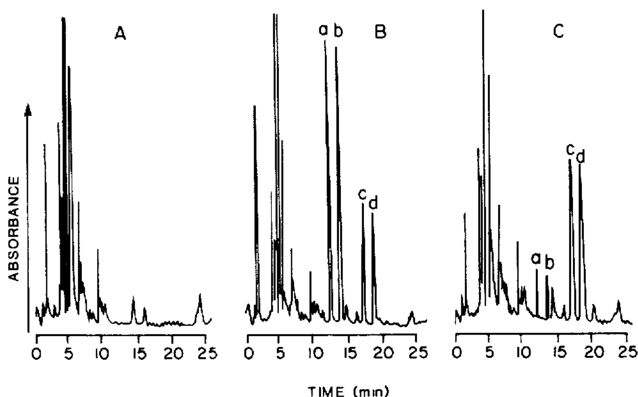

Fig 2 HPLC resolution of the diastereomeric amide derivatives of flurbiprofen extracted from human plasma from a healthy volunteer (A) Blank plasma, (B) plasma spiked with racemic flurbiprofen, (C) 16-h plasma from a patient dosed with 50 mg racemic flurbiprofen Peaks a=S-flurbiprofen, b=R-flurbiprofen, c=S-ibuprofen, d=R-ibuprofen (internal standard)

fluorescence detection was more acceptable than UV because there was less interference from endogenous compounds. However, ibuprofen does not fluoresce well, and consequently was monitored by UV and 232 nm. When flurbiprofen and 3′,4′-OHF were simultaneously quantified in urine the mobile phase was adjusted to acetonitrile–0.05 *M* acetic acid (55 45, v/v) to allow complete resolution of the peaks corresponding to the enantiomers of this metabolite. Fig. 3 illustrates a chromatogram of the derivatives of the dihydroxy metabolite, flurbiprofen, and internal standard (ibuprofen). The lowest level measured was 25 ng/ml for each enantiomer of flurbiprofen and 125 ng/ml for 3′,4′-OHF. A linear response ($r^2 > 0.99$) was obtained for standard curves over the range 25–750 ng/ml for flurbiprofen and 125–750 ng/ml for 3′,4′-OHF. The C V. and relative error were less than 10% in urine both before and after hydrolysis at the 200 and 375 ng/ml level, for example the C V was 4.1, 4 4, 4 8, and 5.9% ($n=8$ for each) for 200 ng/ml *S*- and *R*-flurbiprofen, respectively, in unhydrolyzed and hydrolyzed urine Plasma concentrations of the enantiomers of 3′,4′-OHF were below the limit of detection.

By changing the mobile phase composition, the diastereomeric amide deriv-

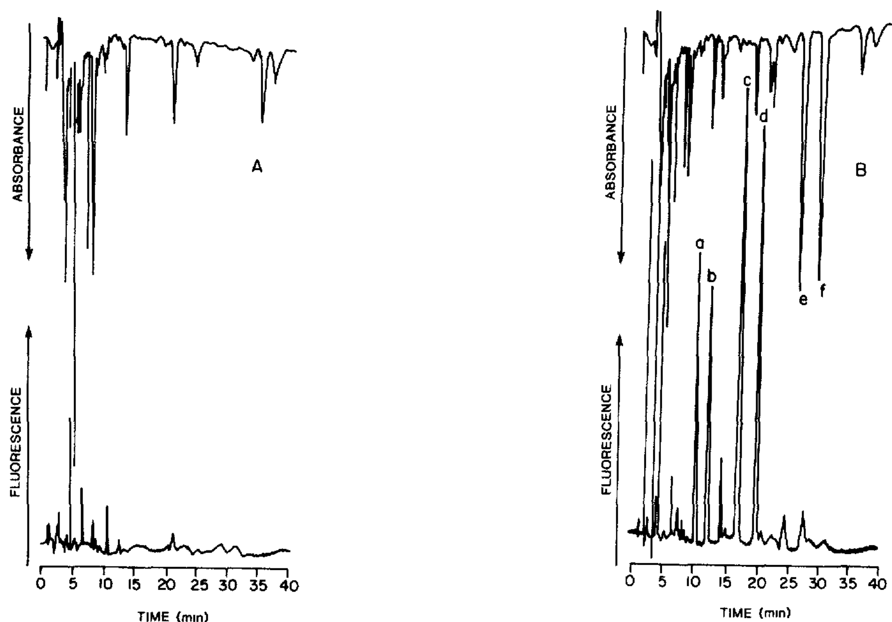

Fig 3 HPLC resolution of the diastereomeric amide derivatives of 3′,4′-dihydroxyflurbiprofen and flurbiprofen extracted from human spiked urine Upper tracing, UV at 232 nm, lower tracing, fluorescence detection (A) Blank urine, (B) urine spiked with racemic 3′,4′-dihydroxyflurbiprofen and racemic flurbiprofen Peaks a=*S*-3′,4′-dihydroxyflurbiprofen, b=*R*-3′,4′-dihydroxyflurbiprofen, c=*S*-flurbiprofen, d=*R*-flurbiprofen, e and f=*S*- and *R*-ibuprofen (internal standard)

atives of the major metabolites of flurbiprofen could also be quantified in plasma and urine. The fluorescence conditions were chosen to obtain a good response for both metabolites which have different fluorescence properties A typical chromatogram of the metabolites in spiked plasma is shown in Fig 4 Analysis of urine obtained from a rat dosed with *S*-flurbiprofen revealed that the *S*-enantiomer of 4′-OHF eluted first followed by *S*-3′-OH-4′-OCH_3, *R*-4′-OHF, *R*-3′-OH-4′-OCH_3, and the internal standard, naproxen The naproxen employed by us consisted of the *S*-isomer alone and therefore a single peak is obtained for this derivative The lowest level measured was 25 ng/ml for 4′-OHF and 75 ng/ml for 3′-OH-4′-OCH_3 The response was linear ($r^2 > 0$ 99) for a concentration range up to 500 ng/ml of each enantiomer The C V and the relative error at 150 and 300 ng/ml of each enantiomer in plasma were less than 10%, for example, the C.V was 2 9 and 4.5% ($n=7$) for 150 ng/ml *S*- and *R*-4′-OHF, respectively, and 6 2 and 5 5% ($n=7$) for 150 ng/ml *S*- and *R*-3′-OH-4′-OCH_3, respectively

The same chromatographic conditions were used to measure the enantiomers of 4′-OHF and 3′-OH-4′-OCH_3 in acid hydrolyzed urine (Fig 5) The standard curve was linear ($r^2 > 0$ 99) up to 750 ng/ml for each enantiomer In acid-hydrolyzed urine the C V and the relative error were less than 10% for both enantiomers of each compound at the 200 and 400 ng/ml level, the C V

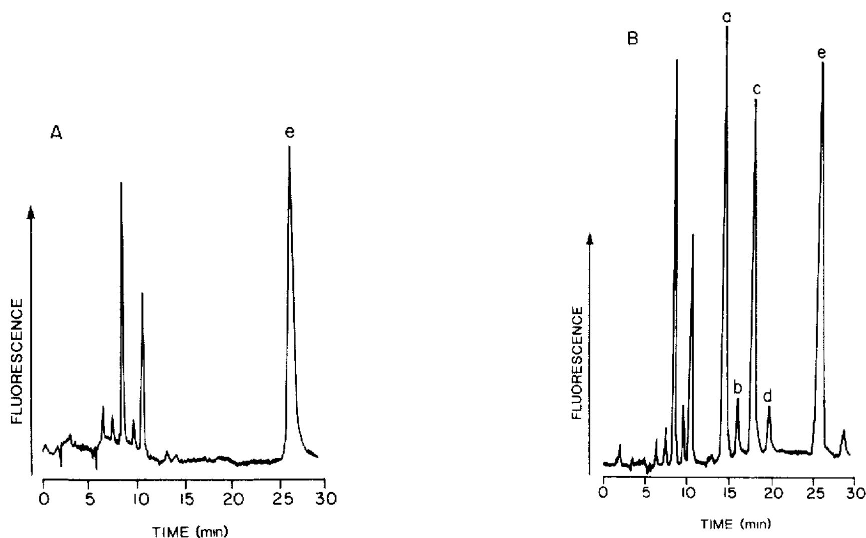

Fig 4 Resolution of the diastereomeric amide derivatives of 4′-hydroxyflurbiprofen and 3′-hydroxy-4′-methoxyflurbiprofen extracted from human plasma (A) Blank plasma spiked with the internal standard, naproxen, (B) plasma spiked with racemic 4′-hydroxyflurbiprofen and 3′-hydroxy-4′-methoxyflurbiprofen Peaks a = *S*-4′-hydroxyflurbiprofen, b = *S*-3′-hydroxy-4′-methoxyflurbiprofen, c = *R*-4′-hydroxyflurbiprofen, d = *R*-3′-hydroxy-4′-methoxyflurbiprofen, e = naproxen (internal standard)

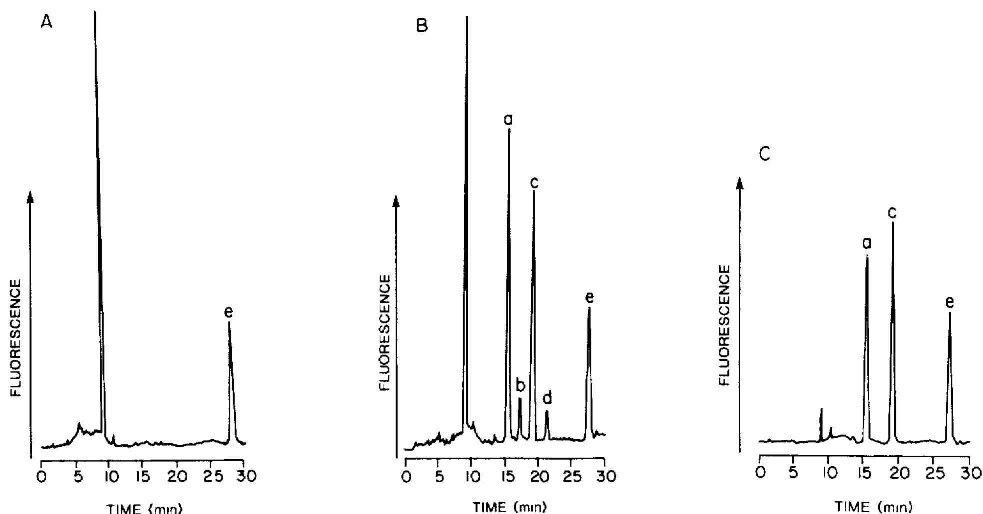

Fig 5 Resolution of the diastereomeric amide derivatives of 4′-hydroxyflurbiprofen and 3′-hydroxy-4′-methoxyflurbiprofen extracted from human urine (A) Blank urine spiked with the internal standard, naproxen, (B) urine spiked with racemic 4′-hydroxyflurbiprofen and racemic 3′-hydroxy-4′-methoxyflurbiprofen, (C) 2 3–2 7 h urine from a patient who received 50 mg racemic flurbiprofen Peak identification as in Fig 4

was 5.9% ($n=10$) for 200 ng/ml of both *S*- and *R*-4′-OHF, and 7 5 and 7 7% ($n=10$ and 9) for 200 ng/ml of *S*- and *R*-3′-OH-4′-OCH_3, respectively

These methods of analysis have been used to measure the plasma and urine levels of the enantiomers of flurbiprofen and metabolites in a healthy volunteer receiving a single oral dose of 50 mg racemic flurbiprofen, plasma and urine samples were obtained over a 24-h period Representative chromatograms from this study are shown for flurbiprofen in plasma (Fig 2C) and the major metabolites in urine (Fig. 5C) The plasma concentration of *S*-flurbiprofen exceeded that of the *R*-enantiomer at all time points, resulting in an *S*/*R* ratio of 1 3 for the area under the curve and 1 2 for the half-life Similar results were obtained by Jamali et al [19] for a single oral dose of 100 mg The latter study employed a convenient assay which resolves the leucinamide derivatives of flurbiprofen, but is significantly less sensitive than our assay and has not been evaluated for metabolite quantitation [15,19] The corresponding urine analysis for our study revealed that 19 7% of the dose was excreted as flurbiprofen and its conjugates, 36 6% as 4′-OHF and conjugates and 3 5% as 3′-OH-4′-OCH_3 and conjugates in 24 h, all with an *S*/*R* ratio of approximately 0 8 The 3′4′-OHF accounted for less than 2% of the dose Comparable data were obtained by Szpunar et al [5] who noted that 22 3% flurbiprofen, 47 6% 4′-OHF, 6 6% 3′-OH-4′-OCH_3, and 1% 3′4′-OHF were excreted in urine primarily as conjugates following a 100-mg oral dose, the corresponding enan-

tiomeric composition was not examined In the study noted above [19] approximately 17% of the administered dose was excreted in urine as flurbiprofen and conjugates with an S/R ratio of 0 8 which is in good agreement with our data.

CONCLUSIONS

Derivatization to form diastereomeric amide derivatives with S-(α)-methylbenzylamine followed by reversed-phase HPLC is suitable for the quantitation of R- and S-flurbiprofen and the R- and S-enantiomers of its major metabolites These methods may provide mechanistic insight into factors which influence the pharmacokinetics of the active S-enantiomer and the degree of enantioselectivity in flurbiprofen disposition

ACKNOWLEDGEMENTS

The authors wish to thank Dr J G Wagner for providing 3′,4′-dihydroxyflurbiprofen. This work was supported by NIH Grants DK37994, DK39546, and AG07631

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