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Stereoselective analysis of fenoprofen and its metabolites

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ABSTRACT

Reversed-phase high-performance liquid chromatographic assays have been developed to quantitate simultaneously fenoprofen and its major metabolites as well as to distinguish between their *R*- and *S*-enantiomers following a single oral dose of 600 mg racemic fenoprofen to healthy volunteers The compounds are extracted from plasma (after precipitation of plasma protein) or assayed directly in diluted urine samples employing a gradient solution on a C_{18} column and ultraviolet detection. Two internal standards, ketoprofen and flunoxaprofen, are used to allow measurement of very low (0 05 μg/ml) and high (70 μg/ml) concentrations in each sample *R*- and *S*-fenoprofen glucuronides can be separated directly, the 4′-hydroxyfenoprofen conjugates are measured via an indirect method by comparing the concentration of 4′-hydroxyfenoprofen before and after hydrolysis. The *R*- and *S*-enantiomers of both parent and 4′-hydroxy metabolite are derivatized with L-leucinamide via an ethyl chloroformate intermediate and subsequently analyzed on a C_{18} column Concentrations of metabolites found in plasma were low when compared to parent drug The *S*/*R* ratio of fenoprofen in plasma always exceeds 1 and increases with time after dosage while the *S*/*R* ratio of its 4′-hydroxy metabolite remains almost unchanged at 1 1. *R*-Fenoprofen glucuronide disappears rapidly from plasma as compared to its *S*-antipode, a less pronounced difference is noted between *R*- and *S*-4′-hydroxyfenoprofen conjugates. Fenoprofen is almost completely excreted as its *S*-acyl glucuronides, the renal clearance of unchanged drug is very low

INTRODUCTION

Formation of acyl glucuronides is a major metabolic pathway for several nonsteroidal antiinflammatory drugs (NSAIDs) and their metabolites [1]. This conjugation involves nucleophilic attack at the C-1 position of the glucuronic acid moiety of uridine diphosphoglucuronic acid (UDGPA) and is catalyzed by membrane-dependent UDP-glucuronyl transferases. For many NSAIDs, the percentage of drug excreted in urine as the glucuronide conjugate is very high, while renal clearance of unchanged drug is low. For example, only small amounts of ketopro-

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fen and naproxen were recovered unchanged in urine [2], and both enantiomers of carprofen exhibited renal clearances of less than 1 ml/min in man [3]. In addition to common phase I and phase II metabolism, the chiral 2-arylpropionic acids may undergo stereoinversion. As reviewed by Hutt and Caldwell [4] and by Jamali [5], significant stereoinversion was found for ibuprofen, fenoprofen, and benoxaprofen.

Except for naproxen, the substituted 2-arylpropionic acids are marketed and dosed as racemic mixtures of *R*-(−)- and *S*(+)-enantiomers. While *in vitro* data showed greater activity for the *S*-(+)-enantiomer, the *in vivo* activities of the enantiomers of some NSAIDs have been shown to be nearly equivalent. *R*-, *S*-, and *R*/*S*-fenoprofen were compared *in vitro* as inhibitors of the fatty acid cyclooxygenase system in human platelets. This system is often used to detect drugs which have antiinflammatory activity that may be associated with inhibition of prostaglandin synthesis [6]. Comparing concentrations causing 50% inhibition of the system, *S*-fenoprofen was estimated to be two times more active than the racemate and about 35 times more active than the *R*-enantiomer. Previous *in vivo* studies in the rat, mouse, and guinea pig showed no differences in pharmacological and toxicological antiinflammatory activities between enantiomers of fenoprofen [7–9]. These differences between *in vitro versus in vivo* results have been attributed to stereoinversion in the body of the relatively inactive *R*-(−)-enantiomer to the more pharmacologically active *S*-(+)-enantiomer [10]. It is proposed that the *R*-isomer is stereoselectively converted to its coenzyme A thioester which is then hydrolyzed by a non-stereoselective racemase to release the *S*-isomer [10,11]. As the *S*-isomer is not a substrate for this enzymatic reaction, the inversion proceeds unidirectionally [4,11,12].

Fenoprofen, α-2-(3-phenoxyphenyl)propionic acid, is a chiral NSAID which undergoes a very extensive *R*- to *S*-inversion in humans [13,14]. Conjugation with D-glucuronic acid *in vivo* results in the formation of glucuronides, which can be separated directly. Furthermore, the parent compound undergoes hydroxylation, forming the diastereomeric metabolite 4′-hydroxyfenoprofen which is also conjugated to its acyl glucuronide (Fig. 1) Precolumn derivatization of fenoprofen enantiomers with L-leucinamide and subsequent resolution of the resultant diastereomers utilizing reversed-phase high-performance liquid chromatography (HPLC) has already been reported [12].

The analytical procedures described here allow simultaneously quantitation of fenoprofen and its metabolites in biological fluids as well as the ability to distinguish between enantiomers of both parent drug and its 4′-hydroxy metabolite following a single oral dose of 600 mg racemic fenoprofen to healthy volunteers.

EXPERIMENTAL

Chemicals

Racemic fenoprofen calcium, 4′-hydroxyfenoprofen, as well as the *R*-(−)- and

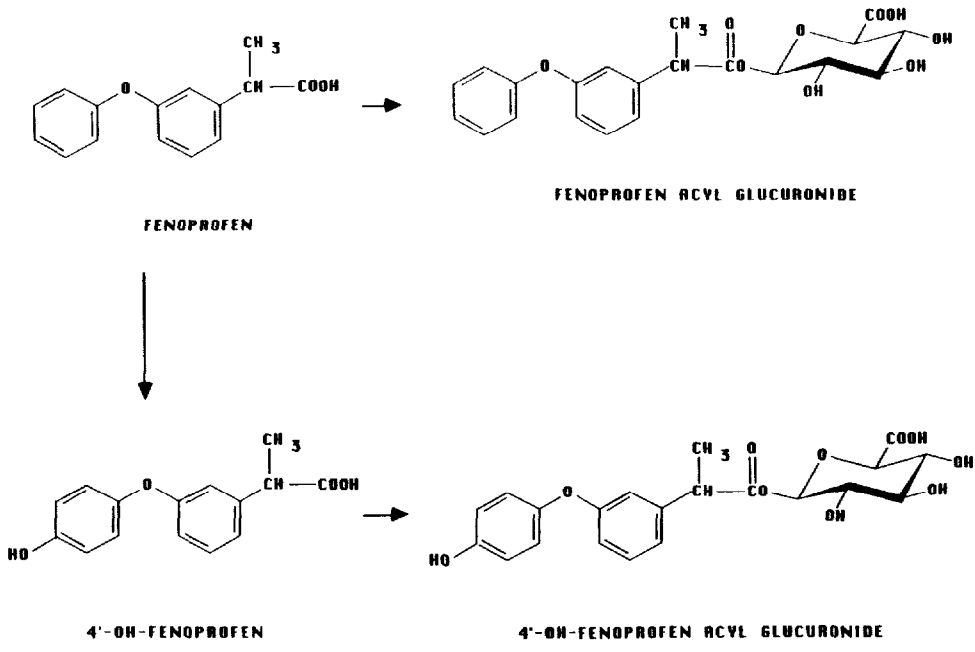

Fig 1 Fenoprofen and its metabolites

S-(+)-enantiomers of fenoprofen were gifts from Eli Lilly (Indianapolis, IN, U.S.A.); enantiomeric purities (formation of diastereomeric amides with α-methylbenzylamine) were for *R* 93.5 ± 0.9% and for *S* 97.5 ± 1.0%. Racemic ketoprofen was obtained from Rhone-Poulenc Sante (Antony, France), and flunoxaprofen from Ravizza (Muggio, Italy). Analytical-grade tetrabutylammonium hydrogensulfate (TBA), potassium phosphate (monobasic), ethyl chloroformate and triethylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.), and L-leucinamide from Fluka (Ronkonkoma, NY, U.S.A.). Acetonitrile, ethyl acetate, and methanol (Fisher, Santa Clara, CA, U.S.A.) were HPLC grade.

Equipment and chromatographic conditions

HPLC analyses were performed using a Beckman gradient system (HPLC programmer Model 420, HPLC pumps Model 110 A; Beckman Instruments, Berkeley, CA, U.S.A.), detection at 272 nm with a Spectroflow 783 UV detector (Applied Biosystems, Ramsey, NJ, U.S.A.), and a 3392 A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). A Beckman Ultrasphere ODS HPLC column (Beckman Instruments) was used as the stationary phase (25 cm × 0.46 cm I.D., 5 μm particle size).

In order to separate the formed metabolites and to remove unconjugated fenoprofen from the column, the mobile phase was adjusted to contain 25% acetonitrile (for 15 min), 32% acetonitrile (for 10 min), and 38% acetonitrile (for 12

min) in 10 m*M* TBA buffer, pH 2 5 (stepwise gradient, flow-rate 1.0 ml/min, temperature ambient). *R*- and *S*-derivatives of fenoprofen and 4′-hydroxyfenoprofen were analyzed using 40% acetonitrile in 60 m*M* potassium phosphate buffer, pH 6 (isocratic).

Human studies

Racemic fenoprofen was administered as a single 600-mg oral dose to six healthy volunteers. Blood samples were taken at 0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, and 24 h after dosage, cooled, and centrifuged. Plasma was separated and immediately adjusted to pH 2–4 (pretreatment of culture tubes with 50 μl of 85% phosphoric acid) to stabilize the ratio of free to conjugated forms of the drug; the samples were stored at −20°C until analysis. Urine was obtained from two volunteers over the time intervals 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–36, 36–48, 48–60, and 60–72 h after administration and immediately acidified after sampling.

Sample preparation

Acidified plasma 1.0-ml aliquots were mixed with 2.0 ml of acetonitrile and centrifuged to precipitate and separate plasma protein. Acetonitrile was evaporated and the samples were extracted with 3 ml of ethyl acetate. After evaporation of the organic solvent to dryness, the residue was reconstituted in 250 μl of mobile phase [containing internal standards ketoprofen (10 μl, 0.125 m*M*) and flunoxaprofen (20 μl, 0.2 m*M*)]. Urine 500-μl aliquots were diluted with 500 μl of mobile phase (containing 50 μl of internal standard ketoprofen 0.25 m*M*) and vortex-mixed.

Quantification of fenoprofen and metabolites in biological fluids

Using the donated fenoprofen enantiomers, the glucuronides of *R*-(−) fenoprofen and *S*-(+)-fenoprofen were prepared *in vitro* using washed sheep liver microsomes as described in ref. 15. Briefly, 2 mg of microsomal protein per ml, 100 m*M* Tris–HCl buffer pH 7.4, 10 m*M* magnesium chloride, 20 m*M* saccharic acid 1,4-lactone, 2 m*M* phenylmethylsulfonyl fluoride, 0.2% Triton X-100, and 1 m*M* substrate (fenoprofen added as a solution in methanol) were preincubated for 5 min at 37°C After addition of 10 m*M* UDPGA, the incubations were continued at 37°C for 45 min. Racemic fenoprofen, 4′-hydroxyfenoprofen, as well as *R*- and *S*-fenoprofen glucuronides were used as reference compounds. Stock solutions of reference compounds and the internal standards ketoprofen and flunoxaprofen were freshly prepared in methanol. Blank plasma and urine were spiked with these standard solutions to establish seven-point calibration curves (concentration range 0–0.5 m*M*; r^2 ranged from 0.995 to 0.999). Some urine sample concentrations exceeded the range of the standard curve and were therefore diluted appropriately and reassayed. Quantitation was based on least-squares linear regression analysis of concentration *versus* peak-height ratio.

Since *R*- and *S*-4′-hydroxyfenoprofen were not available as pure reference compounds and the racemic mixture could not be separated directly on a C_{18} column, the unconjugated compounds (including *R*- and *S*-fenoprofen) were characterized by chiral derivatization. The enantiomeric 4′-hydroxy conjugates were quantified via an indirect method, involving cleavage of the glucuronides and subsequent chiral derivatization This procedure is based on derivatization with L-leucinamide via an ethyl chloroformate intermediate [16,17]. Briefly: to 50 μl of a plasma or urine sample (containing internal standards ketoprofen and flunoxaprofen), 10 μl of 1 *M* sodium hydroxide solution were added, and the conjugates were hydrolyzed at 37°C over 2 h. After reacidification with 10 μl of 1 *M* hydrochloric acid, fenoprofen and 4′-hydroxyfenoprofen were extracted with 1 ml of ethyl acetate, and the organic layer was transferred into a clean tube. After evaporation of ethyl acetate, 100 μl of a triethylamine solution (50 m*M* in acetonitrile) and 50 μl of a ethyl chloroformate solution (60 m*M* in acetonitrile) were added, followed 2 min later by 50 μl of a solution of L-leucinamide (1 *M*) in triethylamine (1 *M* in methanol). Solvents were evaporated, the derivatized products dissolved in 100 μl of mobile phase, and analyzed by HPLC. To another 50 μl of the plasma or urine sample, 20 μl of water were added, and the sample was extracted with 1 ml of ethyl acetate. These extracts were treated as the hydrolyzed samples described above. Glucuronide concentrations were calculated by subtracting the amounts of unconjugated enantiomers from the total enantiomer concentrations. Blank plasma and urine samples spiked with standard solutions of racemic fenoprofen and 4′-hydroxyfenoprofen were extracted, derivatized, and measured using the same analytical procedure as described above. Calibration curves of the derivatives showed linearity up to a concentration of 0.5 m*M* racemate.

RESULTS AND DISCUSSION

Previously reported methods for assaying plasma or urine concentrations of fenoprofen have quantified total parent drug or the enantiomers of parent drug only [13,14]. To our knowledge, no published studies have quantitated fenoprofen metabolites. The HPLC conditions described here allow for simultaneous analysis of the parent drug and its metabolites in plasma and urine samples; furthermore, *R*- and *S*-fenoprofen glucuronides can be separated directly (Fig. 2). Due to their different polarities, fenoprofen and its metabolites could not be conveniently analyzed using a single isocratic system. Applying a gradient system, a sufficient separation was achieved. The lower limit of determination in urine and plasma based on a 100-μl injection volume was 0.1 μg/ml (0.4 m*M*) for fenoprofen and its conjugate and 0.02 μg/ml (0.1 m*M*) for 4′-hydroxyfenoprofen. Since large differences in the concentration ranges between parent drug and metabolites were observed, two internal standards were used so that very low and high concentrations could be measured in each sample. Flunoxaprofen was used

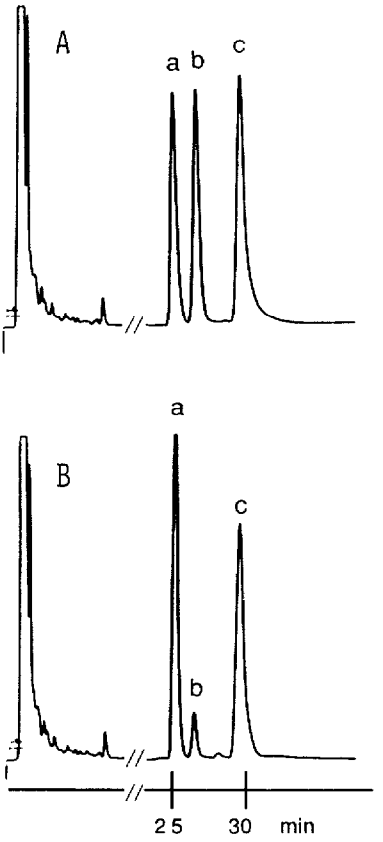

Fig 2. Chromatogram of (A) blank urine spiked with *R*- and *S*-fenoprofen acyl glucuronides, and (B) a urine sample taken from a healthy volunteer 0–2 h after oral administration of 600 mg racemic fenoprofen Peaks a = *S*-fenoprofen glucuronide (t_R = 25 1 min), b = *R*-fenoprofen glucuronide (t_R = 26 3 min), *c* = internal standard ketoprofen (t_R = 29 9 min).

TABLE I

INTRA- AND INTERDAY ASSAY PRECISION OF FENOPROFEN, 4′-HYDROXYFENOPROFEN, AND FENOPROFEN GLUCURONIDE (n = 6)

Compound	Concentration added (μg/ml)	Intra-day C V (%)	Inter-day C V (%)
Fenoprofen	1.25	2.7	4 4
	50	1.7	4 1
4′-Hydroxyfenoprofen	0.05	3.2	6 7
	50	1 9	3 7
Fenoprofen glucuronide	0.05	2 9	5.8
	50	2 2	1.8

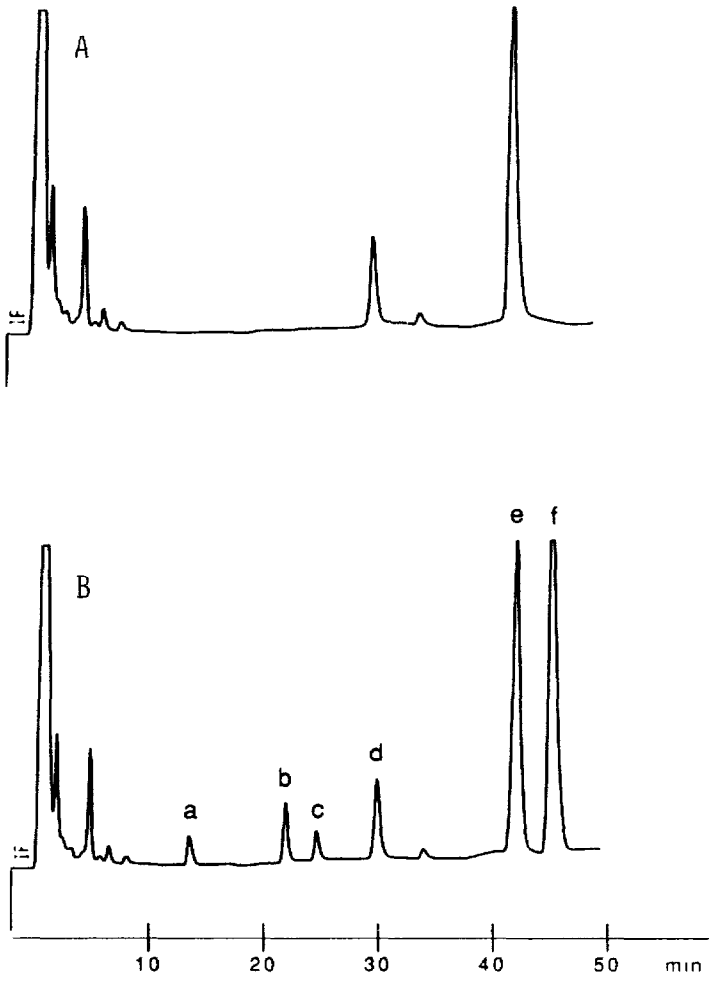

Fig. 3. Chromatogram of (A) blank plasma containing internal standards and (B) a plasma sample 2 h after an oral dose of 600 mg racemic fenoprofen. Peaks a = 4′-hydroxyfenoprofen glucuronide (t_R = 14.8 min), b = 4′-hydroxyfenoprofen (t_R = 21.9 min); c = *S*-fenoprofen glucuronide (t_R = 25.1 min), d = internal standard ketoprofen (t_R = 29 9 min), e = internal standard flunoxaprofen (t_R = 42.2 min), *f* = fenoprofen (t_R = 45 4 min)

as internal standard for fenoprofen, while ketoprofen was used for fenoprofen glucuronide and 4′-hydroxyfenoprofen. The lowest levels routinely quantified were 0.25 μg/ml (1 m*M*) for fenoprofen or its glucuronide and 0.05 μg/ml (0.2 m*M*) for 4′-hydroxyfenoprofen, about twice the minimum limits of determination. Intra- and inter-day coefficients of variation (C.V.) (n = 6) were all less than 7% at low and high concentrations of drug and each metabolite in plasma and urine (Table I). Fig. 3 depicts a chromatogram of fenoprofen and its metabolites

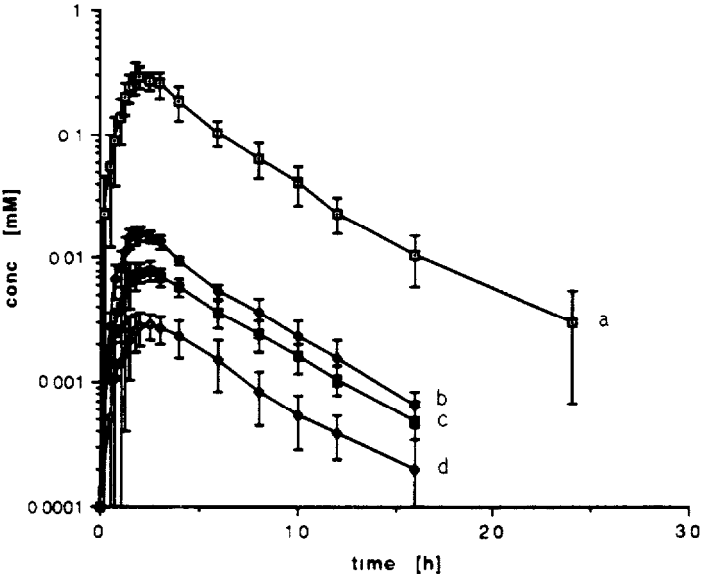

Fig 4 Average plasma concentration–time profiles of fenoprofen and its metabolites in healthy volunteers after an oral dose of 600-mg racemic fenoprofen (n = 6) Error bars indicate standard deviations. (a) Fenoprofen, (b) fenoprofen glucuronide, (c) 4′-hydroxyfenoprofen, (d) 4′-hydroxyfenoprofen glucuronide

extracted from human plasma after a single oral dose of 600 mg fenoprofen. Concentrations of metabolites found in plasma were low when compared to parent drug (Fig. 4). A significant percentage of the dose was excreted into urine as the glucuronides of fenoprofen (51.8%) and 4′-hydroxyfenoprofen (38.7%). Renal excretion of fenoprofen and 4′-hydroxyfenoprofen was low (1.3 and 1.8%, respectively). Fig. 5 depicts an HPLC pattern of human urine after a single 600-mg oral dose of fenoprofen. Total urinary recovery of drug and metabolites accounted for 93.7% of the oral dose.

Since it is known that stereoselective disposition of chiral drugs and metabolites may occur, disposition studies must employ methods that distinguish between enantiomers of both parent drug and metabolites. Derivatization of arylpropionic acids, such as fenoprofen, with L-leucinamide to form diastereomeric amides has been shown to be useful in the separation of these compounds on achiral columns [17]. We have extended this approach to the separation of enantiomers of the metabolite 4′-hydroxyfenoprofen, in addition to fenoprofen. Their diastereomeric conjugates were measured via an indirect method by comparing the concentration of *R*-and *S*-fenoprofen as well as *R*- and *S*-4′-hydroxyfenoprofen before and after hydrolysis. Retention times of the derivatives were 13.6 and 16.0 min for *R*- and *S*-ketoprofen, 21.2 and 23.8 min for *R*- and *S*-flunoxaprofen, 26.5 and 30.0 min for *R*- and *S*-fenoprofen, and 36.6 and 44.2 min

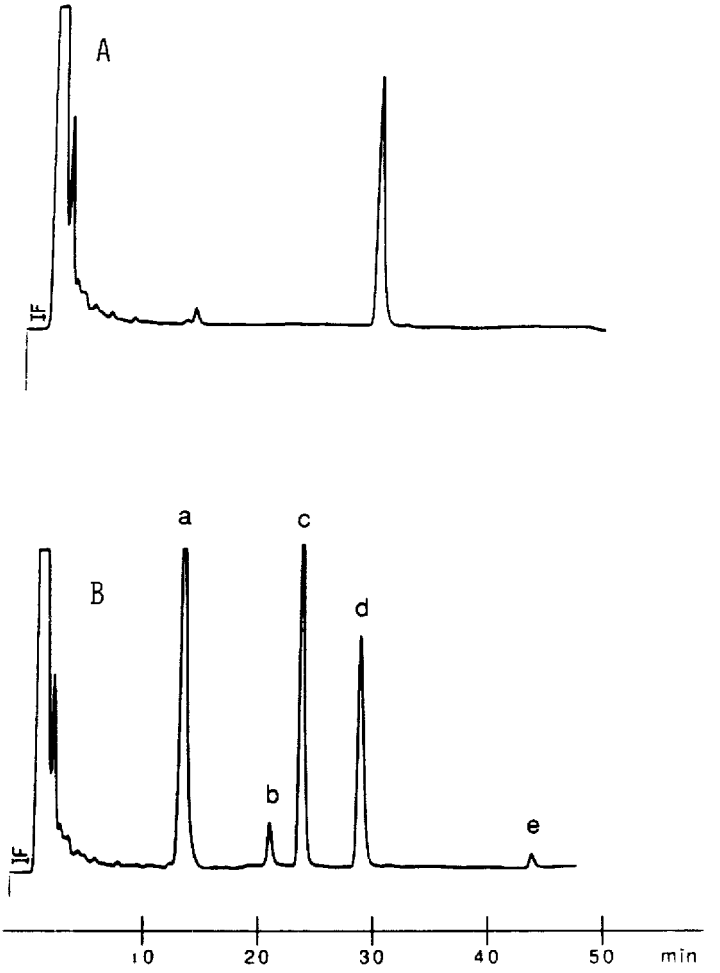

Fig 5 Chromatograms of (A) blank urine containing internal standard ketoprofen and (B) a urine sample 6 h after oral administration of 600 mg racemic fenoprofen Peaks a = 4′-hydroxyfenoprofen glucuronide (t_R = 14 1 min), b = 4′-hydroxyfenoprofen (t_R = 21.0 min), c = fenoprofen glucuronide (t_R = 24 3 min); d = ketoprofen (t_R = 29.1 min), e = fenoprofen (t_R = 44 3 min).

for *R*- and *S*-4′-hydroxyfenoprofen. The *R*/*S* separation and resolution factors for fenoprofen were 1 1 and 3.5, respectively, and for 4′-hydroxyfenoprofen 1.8 and 5.2.

With this chemical chiral derivatization procedure, intra-day coefficients of variation for the derivatives (n= 6) were all less than 7% at low and high concentrations of *R*- and *S*-fenoprofen and its *R*- and *S*-4′-hydroxy metabolite in plasma and urine (Table II). Limits of determination, based on an injection volume of 100 μl, were concentrations of 0.025 μg/ml (0.1 m*M*) for parent drug and 0.063

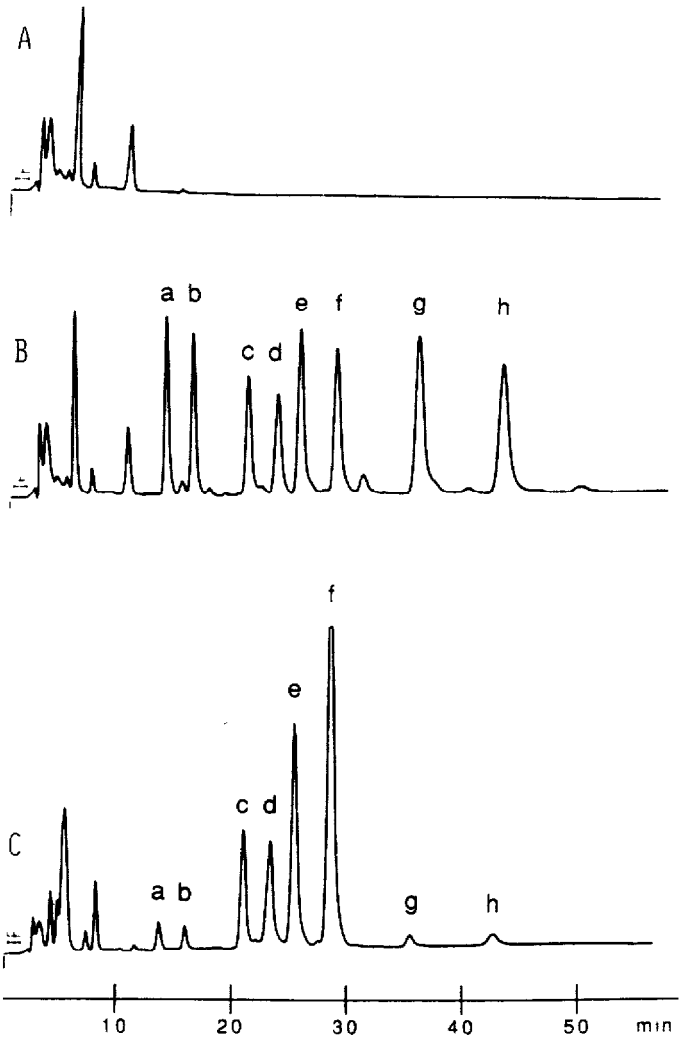

Fig. 6 HPLC resolution of the diastereomeric amide derivatives of fenoprofen and 4′-hydroxyfenoprofen extracted from human plasma. (A) Blank plasma, (B) plasma spiked with racemic internal standards ketoprofen and flunoxaprofen, drug and 4′-hydroxy metabolite; (C) plasma sample from a healthy volunteer obtained 0.5 h after a 600-mg oral dose of racemic fenoprofen Peaks: a and b = *R*- and *S*-ketoprofen derivatives (t_R = 13.6 and 16 0 min), c and d = *R*- and *S*-flunoxaprofen derivatives (t_R = 21 2 and 23.8 min), e and f = *R*- and *S*-fenoprofen derivatives (t_R = 26 5 and 30 min); g and h = *R*- and *S*-4′-hydroxyfenoprofen (t_R = 36 6 and 44 2 min)

μg/ml (0.25 m*M*) for its 4′-hydroxy metabolite. Fig. 6 shows chromatograms of the diastereomeric amide derivatives of the internal standards ketoprofen and flunoxaprofen as well as fenoprofen and 4′-hydroxyfenoprofen.

As demonstrated in previous studies, the plasma concentrations of *S*-fenopro-

TABLE II

INTRA-DAY ASSAY PRECISION OF *R*- AND *S*-FENOPROFEN DERIVATIVES AND *R*- AND *S*-4′-HYDROXYFENOPROFEN DERIVATIVES (n = 6)

Compound	Concentration added (μg/ml)	Intra-day C V. (%)
R-Fenoprofen	1 25	6.5
	50	4.9
S-Fenoprofen	1.25	5.9
	50	5 6
R-4′-Hydroxyfenoprofen	0 125	6 9
	50	5 2
S-4′-Hydroxyfenoprofen	0 125	5 7
	50	5 5

fen exceeded those of the *R*-enantiomer at all times after administration of racemic fenoprofen. Small amounts of *R*-isomer were found in plasma at early time points, but the *S*/*R* ratio always exceeded 1. At the 600-mg dose level, the *S*/*R* ratio approximated 3 at 0.5 h, but by 1 h the ratio had almost doubled, and by 4 h

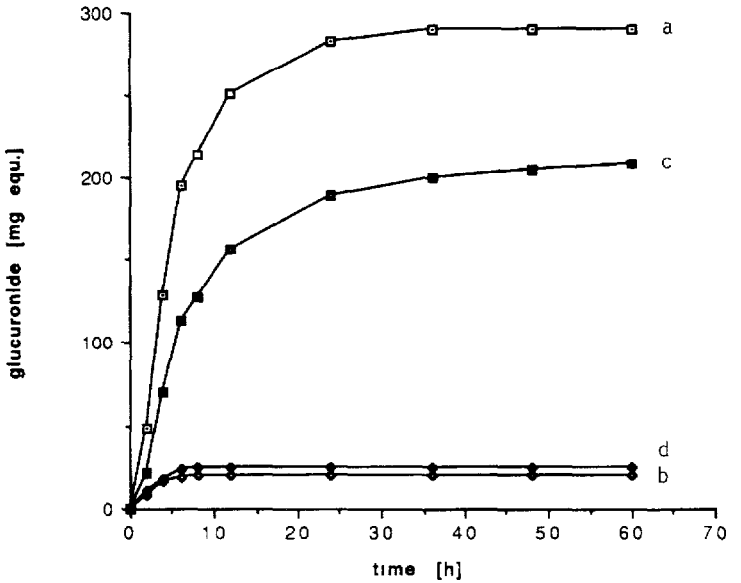

Fig 7. Cumulative excretion of *R*- and *S*-fenoprofen glucuronide (assayed directly) as well as *R*- and *S*-4′-hydroxyfenoprofen glucuronide (quantitated by cleavage and derivatization) in human urine after oral dosage of 600 mg of racemic fenoprofen; average values obtained from two volunteers. (a) *S*-Fenoprofen glucuronide, (b) *R*-fenoprofen glucuronide, (c) *S*-4′-hydroxyfenoprofen glucuronide; (d) *R*-4′-hydroxyfenoprofen glucuronide

no *R*-fenoprofen was detectable. No *R*-enantiomer was detected in hydrolyzed urine [14]. Our results were not significantly different. The measured fenoprofen *S*/*R* ratio in our studies was 2 at 0.5 h and 3 at 1 h after a 600-mg oral dose; concentrations of the *R*-isomer decreased rapidly at later time points. Small quantities of *R*-fenoprofen glucuronide were detected in plasma up to 1 h and in urine up to 8 h after administration of the drug (*S*/*R* ratios were 7 and 11, respectively). In contrast, the *S*/*R* ratio of the metabolite 4′-hydroxyfenoprofen remained almost unchanged at 1.1 in all measured plasma samples; 8 h after drug administration the amount of *S*-4′-hydroxyfenoprofen glucuronide excreted in urine was five fold higher than that eliminated as the *R*-isomer. Due to the very extensive *R*- to *S*-inversion of fenoprofen in humans, *R*-enantiomers of fenoprofen and its glucuronide occur in biological fluids at significantly lower concentrations than their corresponding *S*-isomers. Fig. 7 shows the cumulative excretion of *R*- and *S*-fenoprofen glucuronide as well as *R*- and *S*-4′-hydroxyfenoprofen glucuronide. However, similar *S*/*R* ratios increasing with time after dosage of the racemic drug could not be observed in plasma for the metabolite 4′-hydroxyfenoprofen and its conjugate, although more of its *S*-acyl glucuronide was excreted in the urine.

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