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Evaluation of the stereoselective metabolism of the chiral analgesic drug etodolac by high-performance liquid chromatography☆

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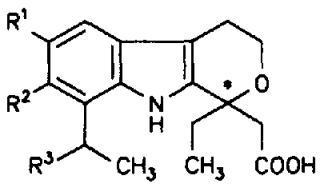
ABSTRACT

The enantiomers of the racemic analgesic drug etodolac have been resolved by fractional crystallization of the diastereomeric salts with optically active 1-phenylethylamine. A high-performance liquid chromatographic method to determine racemic etodolac (assay I) and its major metabolites (assay II) in urine using a conventional reversed-phase column is described. The determination of the enantiomeric ratios of etodolac and the two metabolites 7-hydroxyetodolac and 8-(1′-hydroxyethyl)etodolac was achieved using different protein-bonded chiral stationary phases. The urinary data for five volunteers are presented and show a marked stereoselectivity of the metabolism of etodolac in humans.

INTRODUCTION

Etodolac (1,8-diethyl-1,3,4,9-tetrahydropyrano [3,4-*b*]-indole-1-acetic acid) is a non-steroidal anti-inflammatory drug (NSAID), which is used in the therapy of rheumatic diseases and post-operative pain [1]. Etodolac (1, Fig. 1) is rapidly metabolized to its acyl glucuronide, 6-hydroxy-etodolac (2), 7-hydroxy-etodolac (3), 8-(1′-hydroxyethyl)etodolac (4) and their respective acyl glucuronides. The main route of excretion is the urine [2].

The enantiomers of etodolac have been previously prepared by the formation and HPLC separation of diastereomeric bornyl esters [3], and the *S*-(+)-enantiomer by crystallization with cinchonidine [4] or cholesteryl aniline [5]. The pharmacological effect of etodolac is almost exclusively attributed to the *S*-(+)-enantiomer [3], as described for the 2-arylpropionic acids. A chiral inversion, as reported for ibuprofen [6], is not possible, owing to the chiral cyclic carbon atom.



	R¹	R²	R³
1	–H	–H	–H
2	–OH	–H	–H
3	–H	–OH	–H
4	–H	–H	–OH

Fig. 1. Molecular structures of etodolac (1), 6-hydroxyetodolac (2), 7-hydroxyetodolac (3) and 8-(1′-hydroxyethyl)etodolac (4).

☆ Dedicated to Professor Dr. B. Underhalt on the occasion of his 60th birthday.

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Etodolac displayed a marked stereoselectivity in the pharmacokinetics of its enantiomers [7,8]. In contrast to most other NSAIDs, the plasma level of the active *S*-(+)-enantiomer is higher than the plasma level of the distomer. This has been explained by the higher concentrations of *S*-(+)-etodolac in the synovial fluids [9]. However, a stereoselective metabolic pathway of one enantiomer might also influence the plasma levels. To date, there are no data available about the stereoselectivity of the phase I and phase II metabolism of the enantiomers of etodolac.

This paper describes an economic method for the preparative separation of both enantiomers of etodolac. Furthermore, a HPLC assay to quantify etodolac and its phase I metabolites in human urine after oral application of the racemic drug was developed. The enantiomeric ratio of etodolac, 7-hydroxyetodolac and 8-(1′-hydroxyethyl)etodolac in human urine was determined using chiral stationary phases in HPLC.

EXPERIMENTAL

Chemicals

Etodolac was a gift from Wyeth Pharma (Münster, Germany), and 6-hydroxyetodolac (2), 7-hydroxyetodolac (3) and 8-(1′-hydroxyethyl)-etodolac (4) were obtained from Wyeth Ayerst Research (Princeton, NJ, USA). Acetonitrile, ethyl acetate and 2-propanol were LiChrosolv reagents (Merck, Darmstadt, Germany). The other chemicals were of analytical grade. Buffer solutions were prepared in double-distilled, deionized water and filtered (0.22 μm).

Apparatus

The chromatographic system consisted of a LC-6A liquid chromatograph (Shimadzu, Duisburg, Germany), a Rheodyne sample injector (Model 7125, Rheodyne, Latek, Eppelheim, Germany) equipped with a 20-μl loop, an SPD-6A variable-wavelength detector (Shimadzu), a SCL 6B system controller (Shimadzu) and a C-R6A chromato-integrator (Shimadzu). The UV detector was set at 220 nm, except for the determination of etodolac on the BSA-FA column, which was performed at 230 nm.

Achiral chromatography

The separation of etodolac and its metabolites was achieved with a LiChrospher 100 RP-18 column (5 μm particle size, 250 × 4 mm I.D., Merck, Darmstadt, Germany) with a LiChrospher 60 CN guard column (10 μm particle size, 30 mm × 4 mm I.D.). Eluent I (assay I) was 0.05 *M* phosphate buffer (pH 4.0)–acetonitrile (55:45, v/v). The flow-rate was 1.3 ml/min. Eluent II (assay II) was 0.05 *M* phosphate buffer (pH 4.0)–acetonitrile (73:27, v/v), and the flow-rate 1.2 ml/min.

Chiral chromatography

The enantioseparation of etodolac was achieved on a bovine serum albumin (BSA) stationary phase. The protein was immobilized on silica gel and crosslinked with formaldehyde as described previously [10] (BSA-FA phase, 100 Å pore diameter, 10 Å particle size, 125 × 4 mm I.D.). The mobile phase was 0.05 *M* phosphate buffer (pH 7.0)–2-propanol (93:7, v/v), and the flow-rate 1.0 ml/min.

The enantioseparation of 7-hydroxyetodolac was achieved on a BSA column crosslinked with di-(N-succinimidyl)-carbonate (BSA-DSC phase, 100 Å pore diameter, 10 Å particle size, 125 × 4 mm I.D.) [8]. The mobile phase was 0.05 *M* phosphate buffer (pH 4.0)–2-propanol (98:2, v/v), and the flow-rate 1.2 ml/min.

A Chiral-AGP column (ChromTech, Norsborg, Sweden, 100 × 4 mm I.D.) was used for the separation of the isomers of 8-(1′-hydroxyethyl) etodolac. The mobile phase was 0.005 *M* phosphate buffer (pH 5.5)–acetonitrile (95:5, v/v) and the flow-rate 0.9 ml/min.

A LiChrosorb NH_2 guard column (10 μm particle size, 30 × 4 mm I.D.) was used for all BSA columns.

Preparative separation

A mixture of racemic etodolac (5.2 g, 18.1 mmol) and either *S*-(−)- or *R*-(+)-phenylethylamine (2.1 g, 17.4 mmol) in 30 ml from anhydrous 2-propanol was allowed to crystallize for 12 h at 4°C. Two recrystallization steps for 2-propanol afforded the pure diastereomeric salts. After decomposition of the salts with sulphuric acid

(10%, v/v) and extraction with ethyl acetate, the pure enantiomers of etodolac were obtained and characterized by mass spectrometry, NMR, elemental analysis and melting points (137–138°C). The enantiomeric purity of both enantiomers was at least 98%, as assayed by HPLC analysis on the BSA-FA column.

Determination of etodolac and its ester conjugates in urine by achiral chromatography (assay I)

A 100–200 μl aliquot was diluted with double-distilled water to 1100 μl, and acidified by adding 200 μl of 1 *M* hydrochloric acid. Extraction was performed with 3.0 ml of cyclohexane–ethyl acetate (95:5, v/v). The organic layer was removed and evaporated under a stream of nitrogen. The residue was dissolved in 50 μl of the solution of the internal standard (I.S.) ibuprofen (99.89 μg/ml in acetonitrile), and 20 μl were analysed by HPLC.

The hydrolysis of ester conjugates was performed by the addition of 100 μl of 1 *M* sodium hydroxide (NaOH) and vortex-mixing (2 × 15 s) as described previously [7]. The extraction was performed as described above. The concentration of the conjugated drug was calculated from the difference of the peak areas before hydrolysis (free drug) and after hydrolysis (free and conjugated drug).

Determination of the phase I metabolites and their ester conjugates in urine by achiral chromatography (assay II)

For the determination of 6-hydroxyetodolac (2), 7-hydroxyetodolac (3) and 8-(1′-hydroxyethyl)etodolac (4), 50 μl of the solution of the I.S. suprofen (99.38 μg/ml in acetonitrile) were added to 100–400 μl of urine. The pH was adjusted to *ca.* 3.5 with 1 *M* citrate buffer, and the samples were extracted with 3.0 ml of cyclohexane–ethyl acetate (70:30, v/v). The organic layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of acetonitrile, and an aliquot of 20 μl was injected into the HPLC system.

In order to hydrolyse the ester conjugates of 2–4, the urine was made alkaline with 50 μl of 1 *M* NaOH and vortex-mixed (2 × 15 s) prior to extraction.

Determination of the enantiomeric ratio

For the determination of the enantiomeric ratio of etodolac, urine samples were extracted as described in assay I but without addition of the I.S. The residue was dissolved in 50 μl of 2-propanol, and 20 μl were analysed by HPLC on the BSA-FA column.

The determination of the enantiomeric ratio of 7-hydroxyetodolac (3) and 8-(1′-hydroxyethyl)-etodolac (4), respectively, was achieved after HPLC fractionation of 3 and 4 during the quantitative analysis (assay II). The HPLC fractions were extracted with 3.0 ml of ethyl acetate. The organic layer was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 50 μl of the appropriate mobile phase. The fractions containing 3 were injected into the BSA-DSC column, and the fractions containing 4 into Chiral-AGP. For all determinations the ratios were calculated by means of the peak areas, given by the integrator.

In vivo study

Five healthy volunteers, three male and two female, received 300 mg of etodolac (Lodine) p.o. The urine was collected at 2-h intervals up to 12 h after administration, and then from 12 to 24 h and from 24 to 36 h. The volume and pH were determined immediately, and the samples were stored at −20°C until analysed, at most two months. Acyl glucuronides of NSAIDs are known to be instable [11], but there was no change in the sample concentration during the storage.

RESULTS AND DISCUSSION

Preparative separation

Both enantiomers of etodolac were obtained on a preparative scale by fractional crystallization of the salts of *S*-(−)- and *R*-(+)-1-phenylethylamine, respectively. They were used as reference compounds.

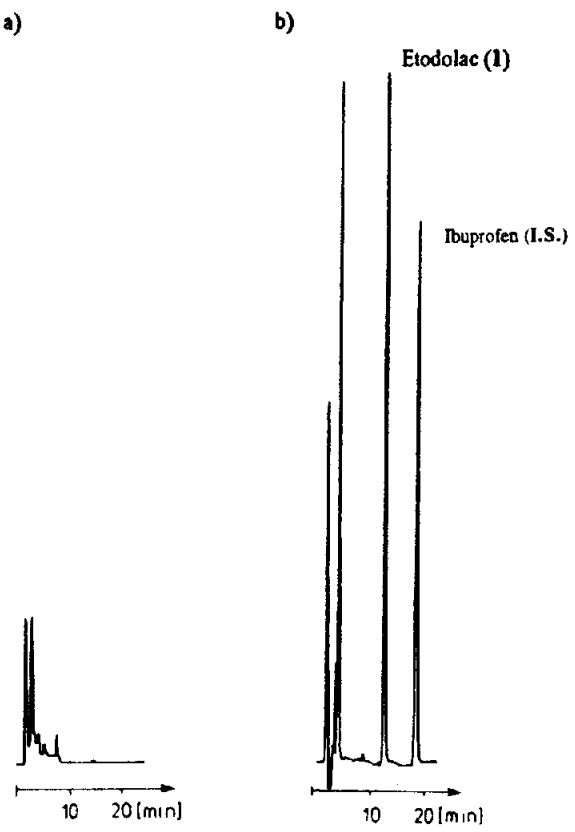

Fig. 2. Chromatograms of (a) a blank urine and (b) etodolac (1) and ibuprofen (I.S.), extracted from hydrolysed human urine after oral administration of 300 mg of etodolac (2–4 h after administration). Chromatographic conditions as in Experimental.

Quantitative determination

Assay I. Fig. 2 shows typical chromatograms of (a) a blank urine and (b) a hydrolysed urine sample after oral administration of racemic etodolac. The retention times were 12.5 min for etodolac and 16.9 min for the I.S., ibuprofen. No interferences were observed after extraction of urine samples. The precision and accuracy of the assay were determined in blank urine samples spiked with known amounts of etodolac, simulating concentrations from 0.125 to 10.0 μg/ml. Each concentration was analysed four times. A linear correlation was found ($y = 0.445\,57x + 0.012\,98$, $r = 0.9999$). Recovery values were evaluated by comparing the peak areas from urine samples spiked with different amounts of etodolac, and those of unextracted standard solutions. The recovery (± S.D.) of etodolac was 93.9 ± 5.3%.

Assay II. In this assay, a mobile phase consisting of 27% acetonitrile in phosphate buffer was used. In order to avoid interfering impurities the urine was buffered to pH 3.5 prior to the extraction. Representative chromatograms of a blank urine sample (a), a urine sample (b) and a hydrolysed urine sample (c) are shown in Fig. 3. The retention times for 2, 3, 4 and the I.S. (suprofen) were 11.2, 15.0, 19.5 and 24.7 min, respectively. Assay precision, reproducibility and calibration curves were determined as described for assay I, the concentrations for calibration curves ranging from 0.24 to 6.38 μg/ml for 6-hydroxyetodolac, from 0.30 to 7.56 μg/ml for 7-hydroxyetodolac and from 0.16 to 4.00 μg/ml for 8-(1′-hydroxyethyl)etodolac (Table II). The equations of the standard curves were $y = 0.391x - 0.025$ for 6-hydroxyetodolac, $y = 0.918x - 0.013$ for 7-hydroxyetodolac and $y = 0.748x - 0.038$ for 8-(1′-hydroxyethyl)etodolac. For all metabolites the coefficient of correlation exceeded 0.9997. The mean recovery (± S.D.) of 6-hydroxyetodolac (2) was 75.8 ± 6.7%, that of 7-hydroxyetodolac (3) was 76.9 ± 6.9% and that of 8-(1′-hydroxyethyl)etodolac (4) was 82.2 ± 7.8%.

Urinary excretion

The cumulative excretion of etodolac and its metabolites is shown for one volunteer in Fig. 4. The concentrations of the metabolites were calculated as milligram equivalents of etodolac. Only negligible amounts of free etodolac (1) and 8-(1′-hydroxyethyl)etodolac (4) were detected in the urine. 7-Hydroxyetodolac (3), its ester conjugates and the ester conjugates of etodolac were the main metabolites in human urine (Table III). The cumulative excretion of these metabolites and their ester conjugates was *ca.* 40% of the administered dose.

In the urine samples of all five volunteers, an additional compound was found (Fig. 3, Met X). Mass spectrometry indicated that this was a further monohydroxylated metabolite of etodolac.

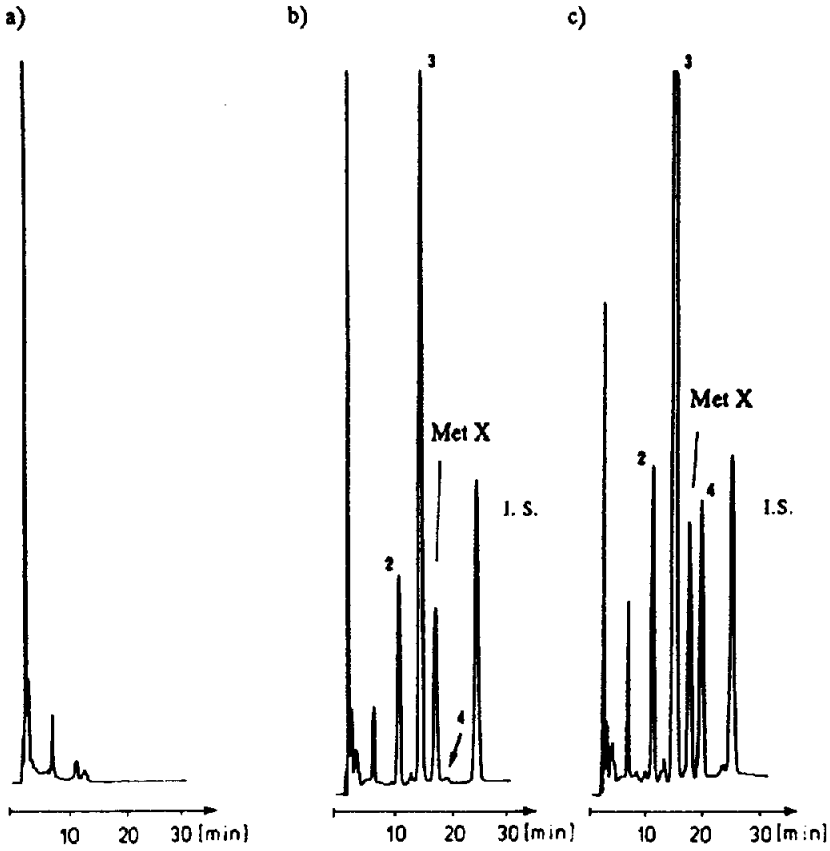

Fig. 3. Chromatograms of (a) a blank urine, (b) a urine sample and (c) a hydrolysed urine sample after oral administration of etodolac (1) (2–4 h after administration). Peaks: 2 = 6-hydroxyetodolac; 3 = 7-hydroxyetodolac; 4 = 7-(1′-hydroxyethyl)etodolac; Met X = new metabolite. Chromatographic conditions as in Experimental.

TABLE I

REPRODUCIBILITY AND RECOVERY VALUES FOR ETODOLAC (ASSAY I)

Values are mean ± S.D.; $n = 4$.

Amount spiked (μg)	Peak-area ratio, 1/ibuprofen	Recovery (%)
0.125	0.057 ± 0.001	102.26
0.251	0.113 ± 0.003	96.87
0.501	0.231 ± 0.009	96.20
0.752	0.361 ± 0.015	96.00
1.002	0.466 ± 0.018	88.04
1.251	0.598 ± 0.010	96.08
2.506	1.126 ± 0.008	86.10
5.012	2.273 ± 0.062	91.40
10.024	4.583 ± 0.021	92.38

Determination of enantiomeric ratios

Jamali *et al.* [7] determined the enantiomers of etodolac indirectly after derivatization with 1-phenylethylamine. We developed a direct method using a BSA-FA column [12]. The excretion procedure was carried out as described for assay I. Because no free etodolac was found in the urine, only hydrolysed samples were analysed. Fig. 5 shows representative chromatograms of a blank urine sample (a) and a hydrolysed urine sample (b). The more polar hydroxylated metabolites did not interfere with etodolac. For calibration, pure enantiomers of etodolac were combined to obtain mixtures of known enantiomeric ratios, in the range from 85:15% to 5:95% (S/R). Each concentration was analysed three times using the

TABLE II

REPRODUCIBILITY AND RECOVERY VALUES (ASSAY II)

Values are mean ± S.D.; $n = 4$.

Amount spiked (μg)	Peak-area ratio, compound/suprofen	Recovery (%)
6-Hydroxyetodolac (2)		
0.239	0.084 ± 0.007	72.71
0.399	0.140 ± 0.007	69.63
0.798	0.276 ± 0.015	73.79
1.595	0.604 ± 0.011	83.01
3.190	1.188 ± 0.009	80.79
6.380	2.492 ± 0.068	75.13
7-Hydroxyetodolac (3)		
0.302	0.233 ± 0.012	71.47
0.567	0.483 ± 0.017	71.87
0.954	0.879 ± 0.024	74.91
1.890	1.743 ± 0.063	76.95
3.780	3.492 ± 0.187	84.71
7.560	6.909 ± 0.303	81.29
8-(1'-Hydroxyethyl)etodolac (4)		
0.160	0.072 ± 0.005	74.51
0.300	0.179 ± 0.011	74.21
0.500	0.351 ± 0.017	83.17
1.000	0.702 ± 0.023	84.97
2.000	1.476 ± 0.025	89.68
4.000	2.974 ± 0.024	86.34

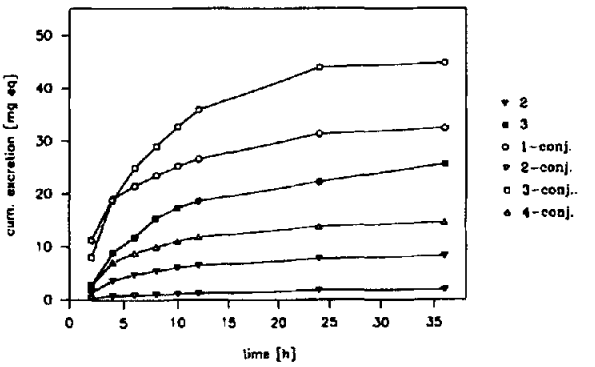

Fig. 4. Cumulative excretion curves of the metabolites of etodolac after oral administration of etodolac to one volunteer. Compound numbering as in Fig. 1.

extraction procedure described above. The recovery of each enantiomer was equal (Table IV).

The separation of 7-hydroxyetodolac can be achieved on the BSA-FA phase, but was improved using BSA crosslinked with di-(N-succinimidyl) carbonate and a mobile phase of phosphate buffer (pH 4.0) with 2% of 2-propanol.

Because of the presence of a second chiral centre, four isomers of 8-(1'-hydroxyethyl)etodolac (4) can exist. The synthetic reference compound 4 is separated into the four stereoisomers on a Chiral AGP® column [13]. Owing to interferences of 7-hydroxyetodolac (3) and 8-(1'-hydroxyethyl)-

TABLE III

EXCRETION OF ETODOLAC AND ITS METABOLITES AFTER ORAL ADMINISTRATION OF ETODOLAC

Values are given as percentages of the administered dose of 300 mg of etodolac. Neither free (non-conjugated) etodolac nor free 8-(1'-hydroxyethyl)etodolac was detected.

Volunteer	Etodolac conjugates	6-Hydroxyetodolac		7-Hydroxyetodolac		8-(1'-Hydroxyethyl)etodolac conjugates
		Free	Conjugates	Free	Conjugates	
1	10.54	1.99	0.56	6.58	12.53	4.61
2	6.43	2.71	0.86	8.35	14.14	4.73
3	10.80	2.74	0.62	8.54	14.91	4.84
4	10.38	2.01	0.32	7.29	12.21	5.27
5	6.77	2.72	0.58	7.99	15.92	4.78

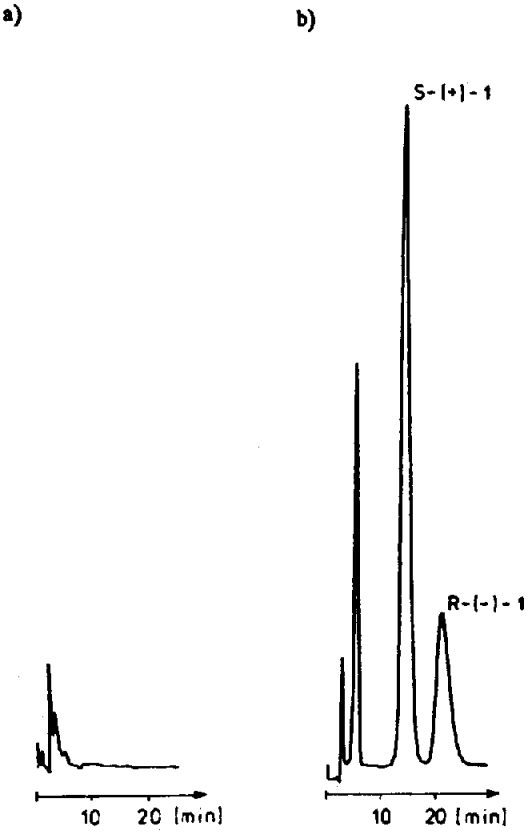

Fig. 5. Chromatograms of (a) a blank urine and (b) the etodolac enantiomers isolated from a hydrolysed urine sample after oral administration of etodolac (2–4 h after administration). Chromatographic conditions as in Experimental.

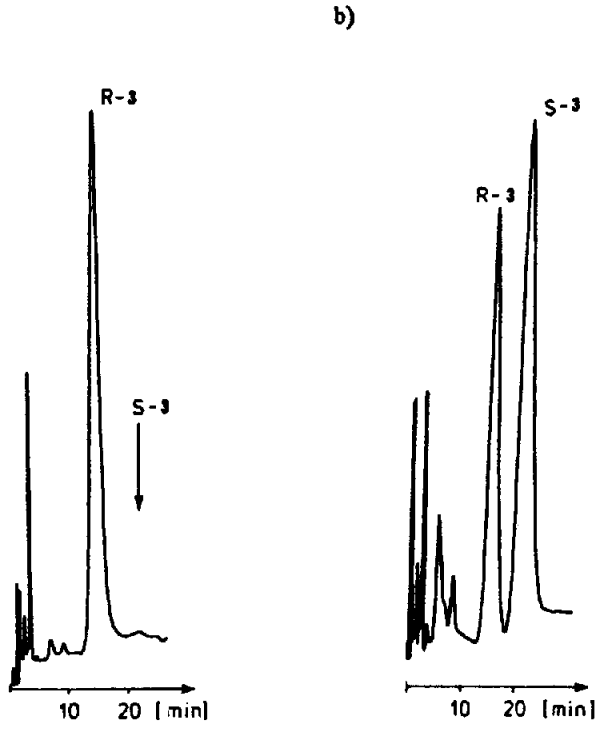

Fig. 6. Chromatograms of the enantiomers of 7-hydroxyetodolac (3) isolated from (a) a urine sample and (b) a hydrolysed urine sample (2–4 h after administration). Chromatographic conditions as in Experimental.

TABLE IV

CALIBRATION OF *S/R* RATIOS FOR ETODOLAC ENANTIOMERS, EXTRACTED FROM URINE (n = 3)

Theoretical (%)		Found (%)		S.D. (%)
S-(+)-1	*R*-(−)-1	*S*-(+)-1	*R*-(−)-1	
85	15	84.51	15.49	0.058
70	30	71.26	28.74	0.144
50	50	50.38	49.62	0.133
30	70	31.18	68.82	0.225
15	85	15.82	84.18	0.450
5	95	4.48	95.52	0.381

etodolac (4) on the chiral stationary phase, 3 and 4 were isolated by HPLC on a reversed-phase column prior to the analysis on the chiral stationary phase. Fig. 6 shows the chromatograms of 7-hydroxyetodolac (3) before and after hydrolysis, isolated from human urine.

In agreement with Jamali *et al.* [7], a marked stereoselectivity in the excretion of the enantiomers of the etodolac conjugates was found. In the first intervals, *S*-etodolac was excreted predominantly, but in later intervals this stereoselectivity was reversed (Fig. 7).

The excretion of free 7-hydroxyetodolac (3) was highly stereoselective (Fig. 8a): the percentage of the *R*-enantiomer ranged from 84% to 97% for all volunteers. In hydrolysed urine samples (free and conjugated 3) a much lower degree of enantioselectivity was found, which also depended on the time elapsed after administration of the drug (Fig. 8b).

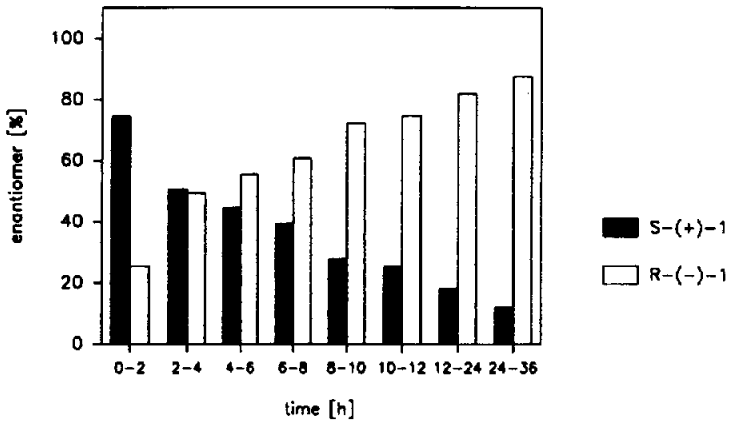

Fig. 7. Enantiomeric ratio of etodolac conjugates in urine after oral administration of racemic etodolac to one volunteer.

In the chromatograms of 4, isolated from hydrolysed urine samples, the last eluting stereoisomer is predominant [12]. This isomer is formed from *S*-(+)-etodolac, as determined by incubation of both etodolac enantiomers with rat liver microsomes [12]. No significant time dependence in the formation of this metabolite was observed.

CONCLUSION

The HPLC assays described are a suitable method for the quantification of etodolac and its metabolites, including the ester conjugates, as well as their enantiomers in human urine. A stereoselectivity in the excretion of the metabolites was found.

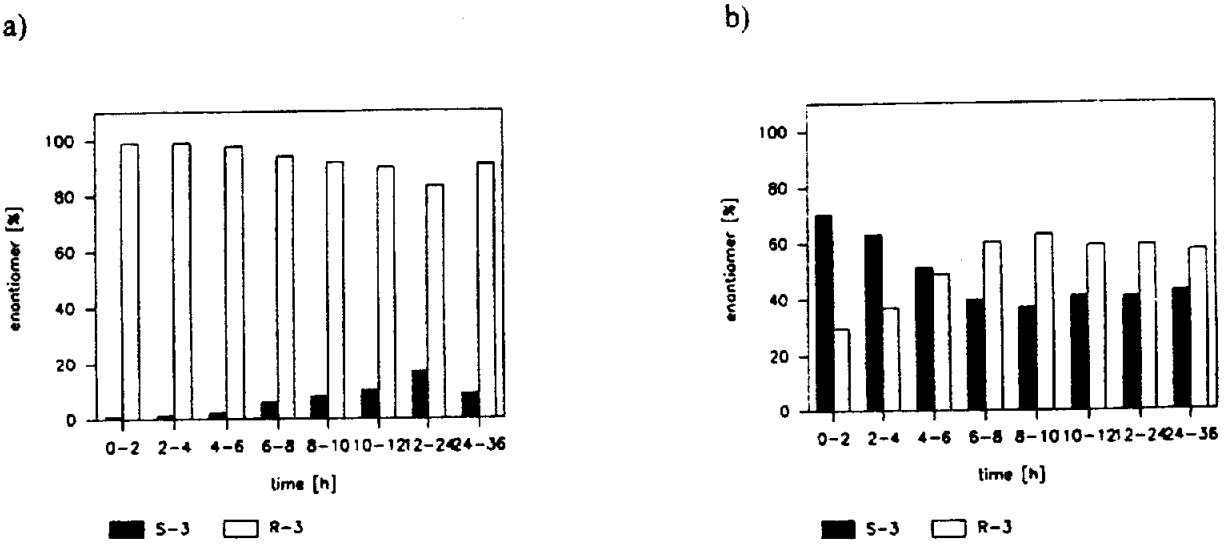

Fig. 8. Enantiomeric ratio of (a) free 7-hydroxyetodolac and (b) total 7-hydroxyetodolac in human urine after administration of racemic etodolac.

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