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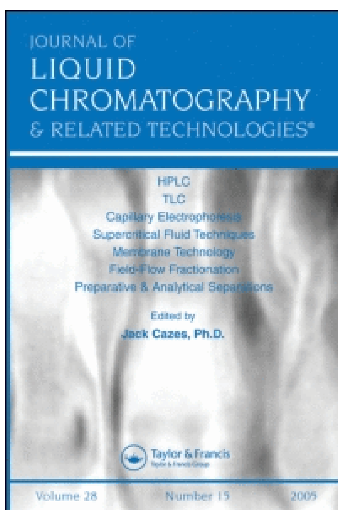

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The Analysis of Ibuprofen Enantiomers in Human Plasma and Urine by High-Performance Liquid Chromatography on an α_1-Acid Glycoprotein Chiral Stationary Phase

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THE ANALYSIS OF IBUPROFEN ENANTIOMERS IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON AN α1-ACID GLYCOPROTEIN CHIRAL STATIONARY PHASE

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ABSTRACT

A method for the quantitation of the enantiomers of the non-steroidal anti-inflammatory drug (NSAID) ibuprofen (IB) in human plasma and urine was developed for pharmacokinetic studies of the individual optical antipodes. Plasma samples were acidified, extracted with organic solvents and analysed by HPLC using an α_1-acid glycoprotein column and UV detection; elution was performed with a phosphate buffer and isopropanol gradient; *RS*-flurbiprofen (FL) was used as internal standard. Calibration curves were linear in the range 0.25 - 25 µg/ml of each IB-enantiomer. Enantiomers and internal standards were baseline separated. Precision and accuracy was ± 3-6%, the limit of detection 0.1 µg /ml, and the analytical recoveries of IB and FL 93.7 ± 5 % and 94.5 ± 4 % resp.; endogenous substances, IB metabolites and other drugs did not interfere with the assay. Urine samples were extracted and analysed as for plasma to assay free, and after alkaline hydrolysis, total IB-enantiomers.

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The described assay is simple to perform, reproducible, accurate and selective for the quantitation of IB-enantiomers in plasma and urine without precolumn derivatisation. Other chiral NSAID drugs were baseline resolved under similar chromatographic conditions: FL, fenopfrofen and ketoprofen; the optical purities of these compounds may be determined with high sensitivity with this HPLC system.

INTRODUCTION

The non-steroidal anti-inflammatory drug (NSAID) ibuprofen (α-Methyl-4-(2-methylpropyl)benzeneacetic acid) (IB) is a widely used drug with analgesic, anti-inflammatory and antipyretic properties; ibuprofen is chiral but is marketed and administered to man as racemic mixtures. In vivo the pharmacologic activity is due mainly to the *S*-(+)-enantiomer and the *R*-(-)-isomer is partially converted with inversion of the configuration at the chiral center to *S*-(+)-IB; this inversion has been proven to be unidirectional (1-3).

It has been suggested that drug stereoselective disposition may be an important factor in the individual therapeutic response to an NSAID (4,5). Kinetics of ibuprofen is complex and cannot be defined satisfactorily in terms of plasma total ibuprofen concentration (6,7); therefore for a proper understanding of stereochemical mechanisms of ibuprofen disposition, it is necessary to measure the IB-enantiomers.

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Aim of the present work was to study the disposition of the ibuprofen enantiomers after analgesic dose administration of racemic ibuprofen in humans under different conditions and in patients with different disease states; for kinetic purpose an assay for the separate determination of the enantiomers in human plasma and urine was developed.

Several assays for the measurement of ibuprofen enantiomers in biological material have been recently described: direct analysis by HPLC with chiral stationary phases (8-10); HPLC after precolumn derivatisation with achiral (11) or chiral reagents (12-14); gas-chromatography - mass spectrometry of IB enantiomers after derivatisation with chiral amines (2,15).

In the present work plasma and urine samples were purified by solvent extraction and the ibuprofen enantiomers were analysed by HPLC using an α1-acid glycoprotein chiral stationary phase and UV detection; total amounts of ibuprofen enantiomers in urine were measured after alkaline hydrolysis; flurbiprofen was used as internal standard.

MATERIALS

Chemicals.

All chemicals were of analytical grade quality. Racemic ibuprofen, flurbiprofen, fenoprofen and

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ketoprofen were available from Sigma (Deisenhofen, FRG); pure *(S)*(+)- and *R*(-)-ibuprofen were gifts from Prof. Dr. U. Klotz, (Dr. M. Fischer-Bosch Institut für Klinische Pharmakologie, Stuttgart, FRG) and from Sepracor Inc. (Marloborough, MA 01752, USA); N,N-dimethyloctylamine (DMOA) (99%) was obtained from Fluka Feinchemikalien (Neu-Ulm, FRG); 2-propanol was Uvasol grade (Merck, Darmstadt, FRG). Solvents for HPLC were filtered and degassed with helium before chromatography.

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Preparation of samples.

a) Volunteers: A single oral 400 mg dose of racemic ibuprofen (Aktren^R, (Bayer AG)) was administered to 18 healthy subjects. For determination of ibuprofen venous blood was collected in heparinized tubes at time intervals till 12 h after administration; the samples were cooled, immediately centrifuged and the plasma was kept frozen at -20°C. Daily 24 h urines were collected during three days and 20 ml aliquots were kept frozen at -20°C. (b) Patients. A single 400 mg oral dose of ibuprofen was administeredTo twelve in-patients with diagnostic liver cirrhosis and blood was collected as described before.

The studies were approved by the Ethics Committee of the Faculty of Medicine, University of Heidelberg.

HPLC equipment.

For the assay of ibuprofen enantiomers a Model 1050 HPLC system consisting of a quaternary pumping system, autosampler, variable wavelength detector and a model 3396 Series II integrator (all from Hewlett Packard GmbH, Waldbronn, FRG) was used; the wavelength was programmed from 0 to 7 min at 220 nm, and from 7 to 15 min at 245 nm. For the determination of possible interferences with other drugs and peak purity tests a HP 1090 A System (Hewlett Packard) with photodiode array detection was used.

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METHODS

Extraction procedure for plasma

1 ml plasma was mixed with 100 μl hydrochloric acid (1 M), 100 μl flurbiprofen (internal standard; 250 μg/ml), 100 μl water and 5 ml ether/hexane (2:8; v/v); the mixture was shaken for 10 min and centrifuged at 900 g for 5 min; 4 ml of the upper organic phase was transferred to a clean tube and evaporated under a stream of nitrogen at 20°C (water bath temperature). Before HPLC analysis, the extract was treated with 100 μl aqueous sodium hydroxide (0.01 M), sonicated for 3

min, followed by 50 μl phosphate buffer (0.1 M, pH 7.0, with 0.1 % dimethyloctylamine) and sonication for 3 min; 5 μl were injected into the HPLC system.

Extraction procedure for urine

a) For the analysis of unconjugated ibuprofen the samples were extracted as described for plasma; b) for the analysis of total ibuprofen (conjugated and unconjugated) 1 ml urine was treated with 500 μl sodium hydroxide (1 M) for 30 min at room temperature; after the addition of 700 μl hydrochloric acid (1 M), 100 μl flurbiprofen (internal standard; 250 μg/ml) and 1 ml water, the mixture was extracted with 5 ml ether/hexane (2:8;v/v) and processed as described for plasma.

Enzymatic hydrolysis with ß-glucuronidase/aryl sulfatase were performed as described (16).

HPLC plasma and urine analysis.

5 μl plasma or urine extract solution was injected into the HPLC system. Column: Chiral-AGP (100 x 4mm I.D., spherical 5 μm particle; ChromTech, Norsborg, Sweden); guard column (10 x 3 mm) filled with the same material. Eluent: solvent (A): 0.01 M aqueous phosphate buffer pH 7.0 modified with 0.001 M

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dimethyloctylamine; solvent (B): 2-propanol modified with 0.001 M dimethyloctylamine / 0.01 M aqueous phosphate buffer pH 7.0 modified with 0.001 M dimethyloctylamine (1:1; v/v). Solvent flow rate: 0.9 ml / min. Gradient elution system 1: 0-5 min isochratic with 0.8% B; 5-15 min linear gradient from 0.8 to 41% B; reequilibration time 10 min; the operating pressure and temperature were 10-12 MPa and 20-25°C respectively. After an analysis series the column was washed with an aqueous 10% 2-propanol solution during 15 min. Peak areas of (*R*) and (*S*) ibuprofen, and peak 2 of the internal standard (Figures 1 and 2) were integrated and the concentrations were calculated using the corresponding calibration factors.

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Preparation of standards.

Plasma: Standard stock solutions of (*RS*) ibuprofen (10.00 mg in 10 ml 0.01 M sodium hydroxide) and (*RS*) flurbiprofen (2.50 mg in 10 ml 0.01 M sodium hydroxide) were prepared and maintained one week in the dark at 2-8°C. The ibuprofen stock solution was diluted with sodium hydroxide (0.01M) to concentrations of 5 to 500 μg / ml. 100 μl of the diluted solutions were added to 1 ml pretreatment plasma to obtain final ibuprofen plasma concentrations of 0, 0.25, 0.5, 1.25, 2.5, 5,

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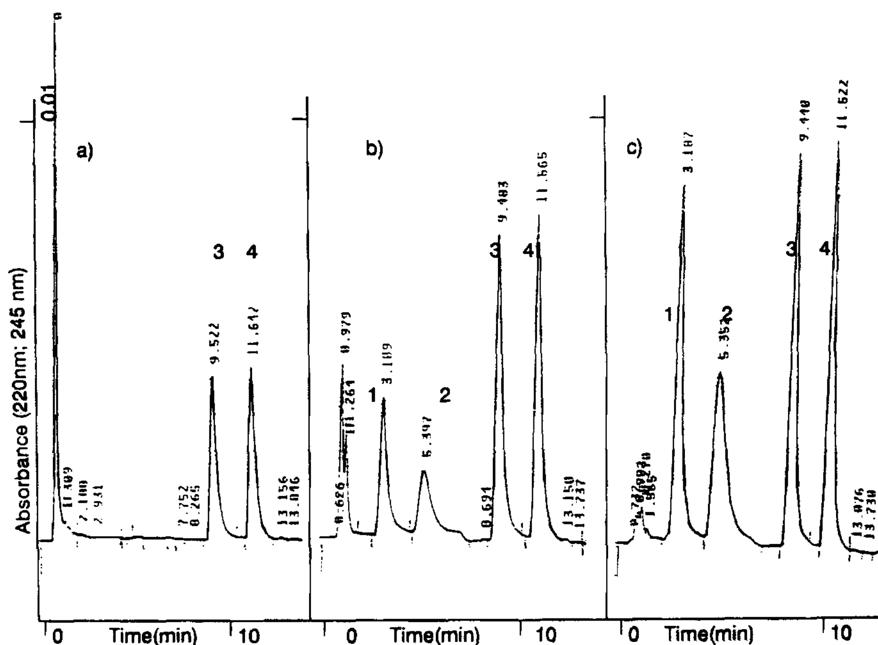

FIGURE 1. HPLC analysis of a plasma extract: a) control plasma; b) control plasma after addition of 10 µg /ml of (*R*) and (*S*) ibuprofen c) plasma sample from a volunteer 45 min after a 400 mg single oral dose of ibuprofen (concentrations of 15.1 and 16.0 µg /ml of (*R*)-and (*S*) -ibuprofen resp.). 25 µg / ml of racemic flubiprofen were added to all samples; 1 and 2 correspond to (R)- and (*S*)-ibuprofen enantiomers; 3 and 4 to the flurbiprofen enantiomers.

12.5 and 25 µg / ml of each enantiomer; after the addition of 100 µl flurbiprofen (internal standard; 250 µg/ml) and 100 µl water, the samples were extracted as described under plasma extraction procedure. *Urine*: The same ibuprofen and flurbiprofen stock solutions and dilutions as described for plasma were used . To 1 ml

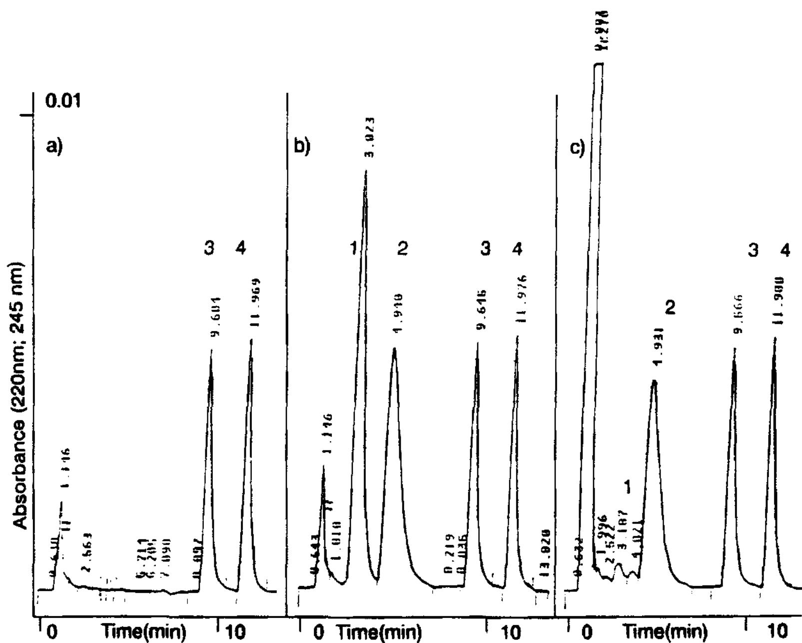

FIGURE 2. HPLC analysis of an urine extract after alkaline hydrolysis: a) control urine; b) control urine after addition of 100 µg / ml of (*R*)-and (*S*)-ibuprofen c) 0-24 h urine sample from a volunteer after a 400 mg single oral dose of ibuprofen (concentrations of 2.0 and 40.2 µg /ml of (*R*)- and (*S*)-ibuprofen resp.); 25 µg / ml of racemic flubiprofen were added to all samples. 1 and 2 correspond to (*R*)- and (*S*)-ibuprofen enantiomers; 3 and 4 to the flurbiprofen enantiomers.

urine, the corresponding amounts of ibuprofen and internal standard were added as for plasma in the ibuprofen concentration range 0 - 100 µg / ml, and processed as described under extraction procedure for urine a) or b).

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Calibration curves.

Calibration curves for plasma and urine analysis were obtained by processing plasma and urine with known amounts of (*R*) and (*S*) ibuprofen and flurbiprofen as mentioned above, before every analysis series. Least-squares linear regression of the ratio of: ((*R*) (or (*S*)) peak area) / (flurbiprofen peak 2 area) vs. added (*R*) (or (*S*)) ibuprofen concentrations were used to calculate the calibration factors.

HPLC separation of NSAIDs enantiomers

1 μl of solutions of ibuprofen, flurbiprofen, fenoprofen and ketoprofen (1 mg / ml in methanol) were analysed by the HPLC equipment and column described above; Gradient elution system 2: linear gradient from 0 to 40% B in 10 mion; reequilibration time 10 min.

RESULTS

Endogenous substances, ibuprofen metabolites, numerous drugs and their metabolites were tested for possible chromatographic interferences (see (16)) and were found not to interfere with the assay; the first peak of ketoprofen showed similar retention times as

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TABLE 1

Column capacity factors (k') and separation factors (α) for the enantiomeric separation of ibuprofen and other NSAIDs. See methods for the description of chromatographic parameters.

Compound	Elution system 1			Elution system 2		
	k'_1	k'_2	α	k'_1	k'_2	α
(RS)-Ibuprofen	1.77	3.24	1.83	1.70	2.08	1.22
(S)-Ibuprofen	---	3.27	---	---	2.16	---
(R)-Ibuprofen	1.75	---	---	1.73	---	---
(RS)-Flurbiprofen	8.26	10.46	1.27	3.36	5.21	1.55
(RS)-Fenoprofen	5.08	7.75	1.52	2.53	2.95	1.16
(RS)-Ketoprofen	3.49	6.38	1.82	2.18	2.57	1.18

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(S)-ibuprofen in both elution systems 1 and 2 (Table 1).

Fig.1 shows chromatograms from plasma extracts: 1a) from pretreatment plasma, 1b) from pretreatment plasma after the addition of 10 μg/ml of each (*R*) and (*S*) ibuprofen, and 1c) from a subject 45 min after administration of an oral dose of 400 mg ibuprofen racemate. Enantiomers were baseline separated: peak assignments for the (R) and (S) antipodes of ibuprofen were obtained by comparing the HPLC retention times after the injection of the pure enantiomers with known absolute configuration (Table 1); pure flurbiprofen enantiomers of known configuration were not obtainable; retention times were constant (± 2%) during an analysis series . A within-run precision of ± 3-6% relative standard deviation was found; the calculated analytical recoveries were 94 % for ibuprofen and 96 % for

flurbiprofen at a concentration of 5 and 12.5 μg / ml of each enantiomer, respectively. Calibration curves were linear in the range 0.25 - 25 μg / ml for each enantiomer. The limit of detection, defined as 3σ above the average blank was 0.1 μg /ml; no racemisation during the procedure could be detected by analysing pretreatment plasma samples after addition of pure (*R*) or (*S*) enantiomers.

Chromatograms from urine samples after alkaline hydrolysis are shown in Fig. 2; Fig 2a of pretreatment sample, 2b) from a pretreatment urine after the addition of 100 μg / ml of each ibuprofen enantiomer,and 2c) shows the chromatogram of a urine sample collected in the first 24 h after ibuprofen administration . The within-run precision, the calculated analytical recoveries, selectivity were similar to plasma. The limit of detection was 0.25 μg / ml and the calibration curves were linear in the range 0.25 - 25 μg / ml for each enantiomer . Total ibuprofen (conjugated and unconjugated) was obtained hydrolysing urine with alkali; this procedure showed higher analytical recoveries of free (*R*) and (*S*) ibuprofen as the enzymatic hydrolysis with ß-glucuronidase / aryl sulfatase and did not show any racemization with samples containing pure (*R*) or (*S*) ibuprofen. The mean cumulative urine elimination of

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total (conjugated and unconjugated) (*R*) and (*S*) ibuprofen in healthy subjects was shown to be 1.17 and 19.0% of the original ibuprofen (R) and (S) dose; unconjugated ibuprofen enantiomers were eliminated only in trace amounts (< 0.5% of the dose).

The peak purities of IB and FL in plasma and urine extracts from subjects after IB administration, were determined by HPLC using a photodiode array detector; the UV spectra of the ascending, apex and descending parts of the curve were superposable with those of the pure compounds.

The enantiomers of racemic fenoprofen and ketoprofen were also resolved by HPLC (Table 1).

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DISCUSSION

The need of analysing large numbers of plasma and urine samples for pharmacokinetic studies on the disposition of ibuprofen enantiomers after racemate drug administration, lead us to reevaluate HPLC assays reported in the literature (8,9,10,11). The method described in the present work used direct HPLC analysis with an α_1-acid glycoprotein column after solvent extraction and showed advantages with respect to the reported ones.

No precolumn derivatisation of ibuprofen with chiral or achiral reagents was necessary; own attempts

to derivatise IB as described in (11) were lengthy and showed interfering artifact peaks originating from side reactions with endogenous substances.

Racemic flubiprofen, of similar chemical structure as IB was used as internal standard for better precision and accuracy; no pure FL enantiomers of known configuration were available for peak assignment. The use of α1-acid glycoprotein as a chiral stationary phase has been reported for the enantiomeric separation of a large number of drugs (17-19, including ibuprofen (9,10). In the present work, IB and the internal standard FL were resolved with base line separation of the enantiomers with good reproducibility and stability; these peaks did not interfere with endogenous substances or other drugs; with careful column handling the column was still useful after more than 800 injections of biological extracts. Experiments with a ß-cyclodextrine phase (8) did not lead to IB enantiomer baseline separation.

The ibuprofen peaks were detected at the absorption maximum 220 nm, and with UV detector time-wavelength programming the internal standard peaks were detected at their absorption maximum at 245 nm; the UV detection was combined with isochratic elution of the ibuprofen peaks, and a gradient elution of the flurbiprofen peaks which allowed a good column selectivity, adequate sensitvity and running times.

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The liquid extraction procedure was simple to perform and showed good analytical recoveries and no chromatographic interferences; low boiling organic solvents were used and the evaporation step was performed at low temperatures as ibuprofen is quite volatile, a fact that is nowhere mentioned; IB extracts must be injected into the HPLC system in aqueous solutions, as band broadening of chromatographic peaks occur when the sample is injected in organic solvents (methanol, isopropanol). Attempts to purify plasma with solid phase extractions of ibuprofen with C_{18} phases showed erratic and non reproducible results.

Potential racemization of IB during the whole analytical procedure was controlled analysing samples containing pure IB enantiomers; no detectable racemization occurred. Pharmacokinetic parameters for plasma were similar to those reported in the literature (11).

The method was also used for the analysis of urine samples; free ibuprofen enantiomers were analysed by direct extraction without hydrolysis; total ibuprofen enantiomers were analysed after alkaline hydrolysis; alkaline hydrolysis showed higher analytical recoveries as the enzymatic hydrolysis with ß-glucuronidase / arylsulfatase and no racemization was detectable; as IB is extensively metabolised by the

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liver (1,2,3) only a fraction of the drug is recoverd in urine , mainly as conjugates (20).

The enantiomers of other chiral NSAID drugs (flurbiprofen, fenopfrofen and ketoprofen) were baseline resolved under similar chromatographic conditions (Table 1): However, the pure enantiomers with known absolute configuration were not available and no peak could be assigned; the optical purities of these compounds may be determined with high sensitivity with this HPLC system.

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(R) and (S) ibuprofen plasma concentrations were measured in patients with liver cirrhosis and in healthy subjects; calculation of pharmacokinetic parameters showed higher average areas under the plasma concentration-time curves ($AUC_{0-\infty}$) (+37% (R), + 27% (S)) and longer plasma elimination half-lives (+40% (R), +44% (S)) for patients with cirrhosis when compared with data from healthy individuals. This indicates an impairment in the elimination of both enantiomers in cirrhosis patients. The mean ratio of ($AUC_{0-\infty}$)(S)/($AUC_{0-\infty}$)(R) was lower in patients than in healthy subjects, indicating an impairment in the inversion of (R) into (S) in patients (21). Similar results were reported by Li et al. (22), who used another assay for the determination of ibuprofen enantiomers.

In summary, a simple, reproducible, accurate and selective method was developed for the analysis of ibuprofen enantiomers in plasma and urine extracts with a direct chiral stationary phase and UV detection was developed for pharmacokinetic studies in humans. The enantiomers of other NSAID may also be separated, and the optical purities of the antipodes determined with high accuracy and sensitvity.

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