Journal of Chromatography, 491 (1989) 139–149
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4711

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF IBUPROFEN, ITS METABOLITES AND ENANTIOMERS IN BIOLOGICAL FLUIDS

G. GEISSLINGER* and K. DIETZEL

Department of Pharmacology and Toxicology, University of Erlangen-Nurnberg, Universitätsstrasse 22, D-8520 Erlangen (F R.G.)

D. LOEW, O. SCHUSTER and G. RAU

PAZ, Arzneimittelentwicklungsgesellschaft mbH, In der Schildwacht 13, D-6230 Frankfurt/Main 80 (F.R G.)

G. LACHMANN

Battelle-Institute e.V., Am Roemerhof 35, D-6000 Frankfurt/Main 90 (F R.G.)

and

K. BRUNE

Department of Pharmacology and Toxicology, University of Erlangen-Nürnberg, Universitätsstrasse 22, D-8520 Erlangen (F.R G)

(First received November 15th, 1988; revised manuscript received January 30th, 1989)

SUMMARY

An isocratic high-performance liquid chromatographic method to determine racemic ibuprofen (assay I) and its major metabolites (assay II) in biological fluids (plasma, urine, bile) using a conventional reversed-phase column is described. A third assay using β-cyclodextrin as stationary phase (Cyclobond I) for the separation of the ibuprofen enantiomers is also described. A wavelength of 220 nm was used to monitor the substances. The sensitivity of the method was 0.1 μg/ml for all three assays. The method was demonstrated to be suitable for stereoselective pharmacokinetic studies of ibuprofen in humans and animals.

INTRODUCTION

Ibuprofen [2-(4-isobutylphenyl)propionic acid] is a non-steroidal anti-inflammatory, analgesic and antipyretic drug. It is widely used in the treatment

0378-4347/89/$03.50

of rheumatic disorders, pain and fever [1]. The metabolism of ibuprofen in humans and several animal species is well documented [2].

The propionic acid side-chain possesses an asymmetric centre, the α-carbon atom. Consequently, two optical isomers, i.e. enantiomers, exist. In vitro, the *S*-(+)-enantiomer exhibits pharmacological effects (inhibition of cyclooxygenase) but the *R*-(−)-form is inactive [3]. In vivo, an inversion of the *R*-(−)-form to the *S*-(+)-isomer occurs in humans and animals. The degree of inversion varies from species to species. Moreover, it has been claimed that the pharmacokinetic behaviour of the two enantiomers in humans may be different [4]. Therefore, the stereoselective investigation of the pharmacokinetic parameters of ibuprofen appears to be of scientific and medical relevance.

Several methods for the quantification of racemic ibuprofen in biological fluids are available [5–13]. Few papers deal with the determination of the main metabolites in urine [10,13,14]. A high-performance liquid chromatographic (HPLC) method for analysing ibuprofen in bile is lacking.

Various methods for the separation of the enantiomers have been published, employing either derivatization to diastereomers by reaction with an optically active reagent (e.g. amines) [15–18] or using optically active stationary phases [19,20].

This paper describes a method to determine racemic ibuprofen (assay I) and its metabolites (assay II) in biological fluids (especially plasma, urine and bile) using a conventional reversed-phase HPLC column. By employing an optically active cyclodextrin column it is possible to separate and quantify both ibuprofen enantiomers (assay III).

EXPERIMENTAL

Chemicals

Ibuprofen and its metabolites (metabolite A = hydroxyibuprofen = 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid; metabolite B = carboxyibuprofen = 2-[4-(2-carboxypropyl)phenyl]propionic acid) were donated by Kanoldt (Höchstadt/Donau, F.R.G.). Flurbiprofen, used as the internal standard (I.S.), was obtained from Eli Lilly (Giessen, F.R.G.). The ibuprofen enantiomers were supplied from Medice (Iserlohn, F.R.G.).

All other chemicals and organic solvents were of HPLC or reagent grade. The mobile phases were freshly prepared and degassed under vacuum, prior to use.

The stock standard solutions of ibuprofen and metabolites were prepared by dissolving an appropriate amount of the compounds in methanol. Working standards were prepared in drug-free plasma, urine or bile from the stock standard to yield concentrations of 1–100 μg/ml of plasma and 10–500 μg/ml of urine and bile, respectively.

Determination of racemic ibuprofen in biological samples (assay I)

The HPLC system consisted of a model 114 M pump (Beckman, Frankfurt/Main, F.R.G.), a Model 165 variable-wavelength detector (Beckman), a Promis (Spark Holland, Emmen, The Netherlands) autosampler and a CR 3A Shimadzu integrator (Egling, F.R.G.). Detection was at 220 nm for all samples and the injection volume was 50 μl. Separation was achieved with a prepacked column (25 cm$\times$4.5 mm I.D., Nucleosil 5-μm RP 8; Bischoff, Leonberg, F.R.G.). The eluent was methanol–water (65:35, v/v), with 1 ml of concentrated phosphoric acid added to each litre. The flow-rate of the mobile phase was 1.6 ml/min.

Determination of hydroxyibuprofen and carboxyibuprofen in biological samples (assay II)

The basic HPLC equipment used in assay II was similar in all respects to that employed in assay I. However, owing to the more polar nature of the two metabolites relative to the unchanged drug, the mobile phase was prepared by combining 270 ml of acetonitrile, 730 ml of water and 1 ml of concentrated phosphoric acid.

Stereoselective determination of S-(+)- and R-(−)-ibuprofen in biological samples (assay III)

The HPLC system used for assay III was similar in all respects to that used in assay I. Separation was achieved with a β-cyclodextrin column (Cyclobond I, Astec, ICT Frankfurt/Main, F.R.G.; 250 mm$\times$4.5 mm I.D. with 5-μm spherical packing). Owing to the greater temperature dependence of the binding constants, we used a column heater set up at 20°C. The mobile phase was acetonitrile–0.1% triethylammonium acetate buffer, pH 7.5 (30:70, v/v). Triethylammonium acetate buffer was made up by taking a 0.1% solution of triethylamine adjusted to pH 7.5 with glacial acetic acid. The usual flow-rate was 0.8 ml/min.

Analytical procedure

Plasma. For the determination of unchanged drug in plasma, a 0.5-ml aliquot was acidified by adding 0.1 ml of 2 *M* hydrochloric acid followed by extraction into 5.0 ml of ice-cooled hexane–diethyl ether (8:2, v/v) containing 25 μg of flurbiprofen as the internal standard by agitating for 15 min at room temperature. After centrifugation for 5 min at 1500 *g*, 4.5 ml of the organic layer were removed and evaporated to dryness under a gentle stream of dry nitrogen. The residue was redissolved in 500 μl of the mobile phase and analysed by HPLC at 220 nm.

The standard curve was prepared by injecting plasma extracts spiked with various amounts of ibuprofen, simulating concentrations from 1 to 100 μg/ml

of plasma. For quantification of unknown plasma samples peak-area ratios for ibuprofen relative to the internal standard flurbiprofen were used.

*Urine and bile.*For evaluation of unchanged drug and metabolites, a 100-μl sample of urine or bile, respectively, was transferred to a screw-topped tube. After acidifying with 150 μl of 1 *M* hydrochloric acid, 5.0 ml of hexane–diethyl ether (8:2, v/v) containing 50 μg of the internal standard were added. After mixing and centrifuging as for plasma samples, 4.5 ml of the upper organic phase were removed and evaporated to dryness. The sample was reconstituted with 500 μl of the mobile phase described for assay II and used for HPLC analysis.

For determination of the total amount of ibuprofen and metabolites (free and conjugated fraction), an alkaline hydrolysis of the urine and bile samples was performed according to Lockwood and Wagner [10]. To a 100-μl fraction of bile and urine, respectively, 50 μl of 1 *M* sodium hydroxide solution were added, followed by an incubation period of 30 min at room temperature with gentle agitation. Afterwards the extraction was performed as described above.

Calibration for the free as well as for the total amount of the compounds was carried out by adding various amounts of ibuprofen and the two metabolites, in the range 50–500 μg/ml urine or bile, to drug-free matrix. These standards were treated in the same manner as the unknown samples. For quantification of unknown samples a calibration curve was prepared by plotting peak areas of the standard samples versus concentrations.

For the analysis of the total amount of the enantiomers of ibuprofen in urine and bile it is not recommended to perform the cleavage of conjugates under alkaline conditions, as alkali may lead to racemization by removing the acidic proton of the α-carbon atom. For this purpose enzymic hydrolysis using β-glucuronidase/arylsulphatase (Boehringer, Mannheim, F.R.G.) was employed. To 100 μl of urine or bile, 100 μl of a 0.3 *M* citrate buffer (pH 5.0) containing 5000 Fishman units/ml were added. The mixture was allowed to incubate in a water-bath maintained at 37 °C for 16 h (overnight) under gentle agitation. Extraction was then performed as described above.

Precision of the assay

Intra-day variation was investigated by adding 1, 5, 20, 40 and 100 μg of ibuprofen to 500 μl of drug-free plasma.

For bile and urine, concentrations of 50, 100, 200 and 500 μg/ml ibuprofen, as well as the metabolites A and B, were employed.

Recovery values

Recovery values were evaluated by comparing extracted spiked samples with unextracted standard solution in mobile phase.

Application

The utility of the methods was demonstrated after oral application of 600 mg of racemic ibuprofen given in capsule form to one human volunteer and intraduodenal administration of 10 mg/kg *R*-(−)-ibuprofen to one beagle dog. In the human experiment, blood and urine were collected over 12 and 24 h, respectively. In the dog study, blood and bile samples were drawn at 30-min intervals. All samples were frozen immediately and stored at −20°C until analysis.

RESULTS

These assays describe a method to quantify total ibuprofen, its enantiomers and the two main phase-I metabolites (hydroxyibuprofen and carboxyibuprofen) in plasma and other biological fluids, e.g. urine and bile, employing only one extraction procedure. The chromatographic resolution was performed using various isocratic HPLC methods.

Assay I

Typical chromatograms of blank plasma, bile and urine samples, as well as a spiked plasma sample, are shown in Fig. 1. Retention times were 10.2 and 12.2 min for flurbiprofen and ibuprofen, respectively. The detection limit was 0.1 μg/ml. Values for the recovery and the precision of the assay are listed in Tables I and II. An excellent linear correlation between peak-area ratios (ibuprofen/I.S.) and ibuprofen concentrations was found ($r=0.9998$).

Assay II

The retention times for hydroxyibuprofen and carboxyibuprofen were 10.1 and 12.2 min, respectively (Fig. 1). Concentrations of 0.1 μg/ml of each metabolite could be detected. Values for the recovery and the precision of the assay are listed in Tables I and II. For both metabolites the coefficients of correlation for the standard curves exceeded 0.998.

Assay III

The enantiomers of ibuprofen, the *R*-(−)- and the *S*-(+)-isomers, were eluted after 27.0 and 29.4 min, respectively (Fig. 1). Less than 2% of each enantiomer in relation to the other could be reliably separated. Analytical recovery values and day-to-day variability of the assay are given in Table I.

The chromatograms presented in Fig. 1 do not show any endogenous substances that would interfere with the analysed compounds.

Standard curves were linear over the investigated concentration range. The correlation coefficients obtained for *S*-(+)-ibuprofen and *R*-(−)-ibuprofen were 0.9990 and 0.9983, respectively.

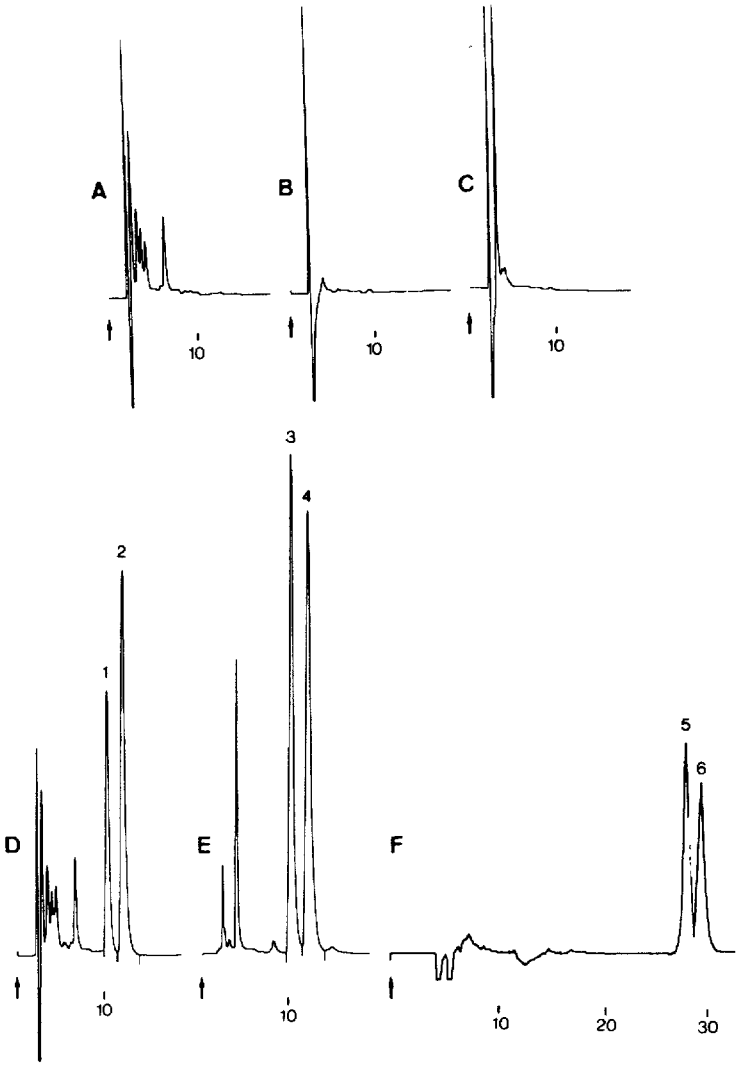

Fig. 1. Chromatograms of ibuprofen, its major metabolites and its enantiomers. (A) Blank human plasma (assay I); (B) blank dog bile (assay I); (C) blank human urine (assay I); (D) human plasma spiked with ibuprofen racemate, 10 μg/ml (assay I); (E) human urine spiked with metabolites A and B, 50 μg/ml (assay II); (F) human plasma spiked with ibuprofen racemate, 10 μg/ml (assay III). Peaks: 1 = internal standard; 2 = ibuprofen; 3 = hydroxyibuprofen (metabolite A); 4 = carboxyibuprofen (metabolite B); 5 = *R*-(−)-ibuprofen; 6 = *S*-(+)-ibuprofen.

Application

Plasma curves and cumulative renal elimination of *R*-(−) and *S*-(+)-ibuprofen (human) are shown in Fig. 2. The urinary excreted amounts of *R,S*-(±)-ibuprofen and metabolites A and B are listed in Table III.

TABLE I

RECOVERY VALUES AND DAY-TO-DAY VARIABILITY OF IBUPROFEN AND ITS ENANTIOMERS IN PLASMA

Values are mean ± S.D.; $n = 6$.

Compound	Concentration (μg/ml)	Concentration found (μg/ml)	Recovery (%)
Ibuprofen (assay I)	1	1.05 ± 0.1	98.5 ± 7.0
	5	4.90 ± 0.2	97.1 ± 5.3
	20	19.2 ± 0.8	98.2 ± 2.8
	40	40.1 ± 1.2	97.3 ± 4.9
	100	98.2 ± 5.1	97.9 ± 3.4
S-(+)-Ibuprofen (assay III)	1	0.98 ± 0.1	96.7 ± 6.8
	2	1.94 ± 0.1	95.9 ± 6.0
	5	5.10 ± 0.3	92.6 ± 4.9
	10	10.0 ± 0.4	98.3 ± 3.6
R-(−)-Ibuprofen (assay III)	1	0.96 ± 0.1	97.1 ± 6.1
	2	2.05 ± 0.2	100.0 ± 5.8
	5	4.90 ± 0.2	96.3 ± 5.1
	10	9.90 ± 0.4	94.9 ± 4.1

TABLE II

RECOVERY VALUES AND DAY-TO-DAY VARIABILITY OF IBUPROFEN AND METABOLITES IN URINE AND BILE

Values are mean ± S.D.; $n = 6$.

Compound	Concentration (μg/ml)	Concentration found (μg/ml)		Recovery (%)	
		Urine	Bile	Urine	Bile
Ibuprofen (assay I)	50	51.8 ± 4.1	47.9 ± 3.9	93.2 ± 4.3	96.8 ± 5.8
	100	94.6 ± 8.6	99.7 ± 6.1	95.3 ± 3.7	94.3 ± 3.8
	200	197.9 ± 10.1	200.7 ± 8.9	92.2 ± 4.8	94.7 ± 3.1
	500	501.3 ± 19.8	500.4 ± 13.6	97.1 ± 3.4	93.5 ± 4.5
Hydroxyibuprofen (assay II)	50	48.7 ± 3.5	49.3 ± 3.3	90.1 ± 4.5	89.9 ± 1.7
	100	98.5 ± 4.9	100.8 ± 5.8	92.2 ± 4.0	86.2 ± 3.1
	200	195.6 ± 6.5	199.3 ± 11.6	94.3 ± 5.4	87.1 ± 3.6
	500	508.9 ± 26.8	504.5 ± 26.0	87.2 ± 6.1	85.2 ± 3.5
Carboxyibuprofen (assay II)	50	43.1 ± 2.3	49.1 ± 2.7	92.2 ± 3.1	90.0 ± 4.2
	100	90.6 ± 8.5	99.3 ± 8.8	90.3 ± 4.4	92.5 ± 5.0
	200	194.2 ± 10.7	193.6 ± 21.5	88.3 ± 5.6	91.2 ± 6.1
	500	503.7 ± 25.6	508.9 ± 22.9	86.1 ± 5.9	87.3 ± 3.8

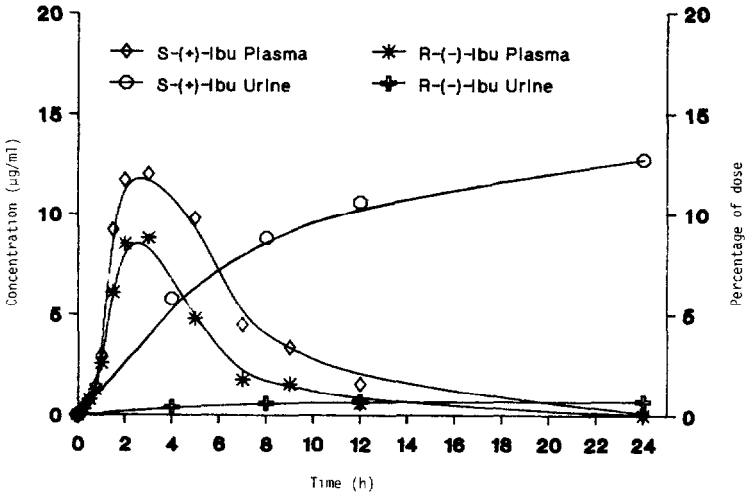

Fig. 2. Plasma concentration–time curves and cumulative renal excretion of the ibuprofen enantiomers after oral administration of 600 mg of ibuprofen racemate to a volunteer.

TABLE III

RENAL EXCRETION OF IBUPROFEN AND METABOLITES AFTER ORAL APPLICATION OF 600 mg OF RACEMIC IBUPROFEN

Values are percentage of dose.

Time (h)	Rac. ibuprofen[a]		*R*-(−)-Ibuprofen[b]		*S*-(+)-Ibuprofen[b]		Metabolite A[c]		Metabolite B[c]	
	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total
0- 4	0.1	6.3	0.0	0.4	0.1	5.8	1.1	3.8	5.6	7.6
4- 8	0.1	3.1	0.0	0.2	0.1	3.0	2.3	4.8	8.8	10.9
8-12	0.1	1.9	0.0	0.1	0.1	1.8	1.2	2.9	5.7	6.3
12-24	0.1	2.2	0.0	0.0	0.1	2.1	3.6	7.8	14.1	16.7
0-24	0.4	13.5	0.0	0.7	0.4	12.7	8.2	19.3	34.2	41.5

[a]By assay I.
[b]By assay III.
[c]By assay II.

Plasma curves and cumulative biliary excretion of *R*-(−)- and *S*-(+)-ibuprofen (beagle) are demonstrated in Fig. 3.

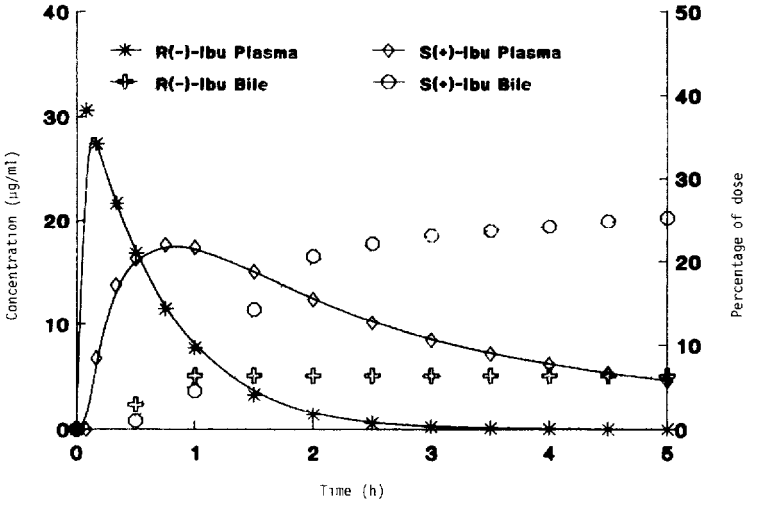

Fig. 3. Plasma concentration–time curves and cumulative biliary excretion of the ibuprofen enantiomers after intraduodenal application of 10 mg/kg *R*-(−)-ibuprofen to a beagle dog.

DISCUSSION

The method presented, consisting of a one-step extraction and isocratic HPLC analysis, appears to be suitable and easy to handle for the quantification of racemic ibuprofen, its enantiomers and phase-I metabolites in biological samples, e.g. plasma, bile and urine.

For the separation of enantiomers, several methods are conceivable: (1) synthesis of diastereomeric derivatives by reaction with an optically active reagent (e.g. amines) followed by chromatography on a reversed-phase column [15–18]; (2) the use of a chiral mobile phase; (3) the use of a chiral stationary phase [19,20].

All these methods have certain disadvantages: they take too long, or the reaction of the drug with the derivatizing chiral agent is too variable, or the cost of the optically active materials is too great.

Previous work on separating the ibuprofen enantiomers employed preferentially diastereomeric synthesis, e.g. Lee et al. [15] described the esterification of the ibuprofen antipodes with *S*-(+)-2-octanol.

The use of a chiral mobile phase seems likely to be very expensive.

Several chiral stationary phases have recently become available [19,20]. Hermansson [21,22], for example, developed a chiral HPLC column based on human plasma protein (α_1-acid glycoprotein, orosomucoid); this column requires the use of aqueous buffer solutions.

Another commercially available optically active column is the β-cyclodextrin (β-CD) bonded phase column [23]. This macrocyclic molecule contains

seven glucopyranose units arranged in the shape of a hollow truncated cone, in which the interior cavity is relatively hydrophobic. The exterior faces, on the other hand, are hydrophilic [24]. Because of the rigid cavity size of β-CD, only 'guest' molecules of proper size can form strong β-CD inclusion complexes. If optimized pH values and salt concentrations are used with the mobile phase, sufficient separation of the optical isomers of ibuprofen is possible.

It has been demonstrated that pH is one of the most effective separation parameters. We found that triethylammonium acetate buffer of pH 7.5 allows a good separation (Fig. 1), provided the injected amount of ibuprofen does not exceed 0.5 μg. Samples with higher amounts should be adequately diluted with mobile phase. Further improvement of separation would seem to be possible. It may be achieved by testing other solvent ratios.

The most suitable column had to be selected from several production batches because the separation power, with respect to the enantiomers, turned out to be related to the individual columns used.

Nevertheless, the assay proved to be sufficiently precise and sensitive, as indicated by comparison of the sum of the concentrations of the two enantiomers and the amount of racemic ibuprofen as found by assay I. We found a good conformity over the whole concentration range investigated.

Using assays I, II and III it was possible to investigate intensively and accurately the pharmacokinetics of ibuprofen.

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