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Stereospecific high-performance liquid chromatographic analysis of ibuprofen in rat serum

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Abstract

A simple, rapid and sensitive high-performance liquid chromatographic method was developed for determination of ibuprofen, (±)-(*R, S*)-2-(4-isobutylphenyl)-propionic acid, enantiomers in rat serum. Serum (0.1 ml) was extracted with 2,2,4-trimethylpentane/isopropanol (95:5, v/v) after addition of the internal standard, (*S*)-naproxen, and acidification with H_2SO_4. Enantiomeric resolution of ibuprofen was achieved on ChiralPak AD-RH column with ultraviolet (UV) detection at 220 nm without interference from endogenous co-extracted solutes. The calibration curve demonstrated excellent linearity between 0.1 and 50 μg/ml for each enantiomer. The mean extraction efficiency was >92%. Precision of the assay was within 11% (relative standard deviation (R.S.D.)) and bias of the assay was lower than 15% at the limit of quantitation (0.1 μg/ml). The assay was applied successfully to an oral pharmacokinetic study of ibuprofen in rats.

Keywords: Enantiomer separation; Ibuprofen

1. Introduction

Ibuprofen, (±)-(*R, S*)-2-(4-isobutylphenyl)-propionic acid, is a chiral 2-arylpropionic acid non-steroidal anti-inflammatory drug (NSAID) (Fig. 1). Ibuprofen has been utilized since 1969 in the UK and since 1974 in the North American market and demonstrates stereoselectivity in its pharmacokinetics [1]. There is renewed interest in the use of non-steroidal anti-inflammatory drugs including the enantiomers of ibuprofen in the treatment of a variety of cancers including prostate and colon cancer [2].

Since 1975, ibuprofen has been chromatographed utilizing a variety of indirect and direct stereospecific methods. Methods of analysis of ibuprofen enantiomers have previously been reviewed [3,4]. Several of the methods reported for derivatization require extensive sample preparation, long derivatization, lengthy chromatography times, poor sensitivity, and late eluting peaks [5–9]. Formation of disatereomeric derivatives may introduce inaccuracies into the determination of enantiomeric ratio due to chiral

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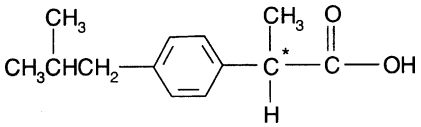

Fig. 1. Structure of ibuprofen. The asterisk denotes chiral carbon.

impurities in the reagent or to the racemization during the process of derivatization [10,11]. Direct enantiomeric analysis using enantioselective chiral stationary phases avoids these problems. There are only a few validated direct methods of stereospecific analysis of ibuprofen despite some concerns over stereochemical conversion of ibuprofen enantiomers during indirect methods [3,4]. In the case of high-performance liquid chromatography (HPLC), many of the columns could previously only be used with non-aqueous solvents. The ChiralPak AD-RH column is a newly commercially available tris(3,5-dimethylphenylcarbamate) derivative of amylose, which can be utilized in the reverse-phase mode. To our knowledge, no validated assay method has been published characterizing the separation of NSAID enantiomers using this column. However, ibuprofen has been reported to be resolved on this column using reversed-phase solvents [12].

The present study describes a stereospecific, isocratic, reversed-phase high-performance liquid chromatographic method for the determination of the enantiomers of ibuprofen in serum and its application to pharmacokinetic studies in rats.

2. Experimental

2.1. Chemicals and reagents

Racemic (*R*, *S*)-ibuprofen, (*S*)-naproxen (purity 98%) and (*S*)-ibuprofen (purity >99%) and triethylamine were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, 2-propanol, and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). 2,2,4-Trimethylpentane, sulfuric acid and phosphoric acid 85% were from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Sprague–Dawley rats were obtained from Charles River Laboratories. Ethics approval for animal experiments was obtained from Washington State University.

2.2. Chromatographic system and conditions

The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT VP pump, an SIL-10AF auto-injector, an SPD-M10A VP spectrophotometric diode-array detector, and a SCL-10A VP system controller. Data collection and integration were accomplished using Shimadzu EZStart 7.1.1 SP1 software (Kyoto, Japan).

The analytical column used was a ChiralPak AD-RH column (150 mm × 4.6 mm i.d., 5 μm particle size, Chiral Technologies Inc., Exton, PA, USA). The mobile phase consisted of acetonitrile, water, phosphoric acid, and triethylamine (35:64.85:0.1:0.05, v/v/v/v), filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (25 ± 1 °C), and a flow rate of 0.8 ml/min, with ultraviolet (UV) detection at 220 nm.

2.3. Stock and working standard solutions

An amount of 25 mg of racemic ibuprofen was accurately weighed on an analytical balance (AG245, Mettler) and dissolved with methanol in a 25 ml volumetric flask to make a stock standard solution in methanol with a concentration of 1 mg/ml of racemic ibuprofen. A methanolic stock solution of (*S*)-naproxen (internal standard) was prepared similarly with a concentration of 1 mg/ml. This solution was diluted with methanol to make a working internal standard solution of 20 μg/ml. These solutions were protected from light and stored at −20 °C between use, for no longer than 3 months. Calibration standards in serum were prepared daily from the stock solution of ibuprofen by sequential dilution with blank rat serum, yielding a series of concentrations of the individual enantiomers, namely, 0.1, 0.25, 0.5, 1, 5, 10, 25 and 50 μg/ml, in six replicates.

Quality control (QC) samples were prepared from the stock solution of racemic ibuprofen by dilution with blank rat serum to yield concentrations of the individual enantiomers of 0.1, 0.25, 5 and 50 μg/ml. The QC samples were divided into 0.1 ml aliquots in microcentrifuge tubes and stored at −70 °C before use.

2.4. Sample preparation

To the working standards or samples (0.1 ml) were added 20 μl of internal standard solution

(20 μg/ml), 40 μl of 0.6 M sulfuric acid, and 1 ml of 2,2,4-trimethylpentane/isopropanol (95:5, v/v). The mixture was vortexed for 1 min (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 3500 × *g* for 5 min (Fisher Marathon 16KM Microcentrifuge, Fisher Scientific, Pittsburgh, PA, USA). The organic phase was collected and evaporated to dryness using a Heto Vac concentrator (Heto-Holten, DK-3450 Allerød, Denmark). The residue was reconstituted with 100 μl of 70% (v/v) methanol, vortexed for 1 min and centrifuged at 2500 × *g* at 4 °C for 5 min, and 50 μl of the supernatant was injected onto the column.

2.5. Precision and accuracy

The within-day precision and accuracy of the assays performed in replicate ($n = 6$) were tested by using four concentrations of the individual enantiomers, namely, 0.1, 0.25, 5 and 50 μg/ml. The between-day precision and accuracy of the assays were estimated from the results of six replicate assays on six different days within 1 week. The precision was evaluated by the relative standard deviation (R.S.D.). The accuracy was estimated based on the mean percentage error of measured concentration to the actual concentration [13].

2.6. Recovery

Recovery for ibuprofen enantiomers from rat serum was assessed ($n = 6$) at 0.1, 0.5, 5 and 50 μg/ml. The recovery of the internal standard, (*S*)-naproxen, was evaluated at the concentration used in sample analysis (4 μg/ml). Ibuprofen or (*S*)-naproxen was spiked into 0.1 ml rat serum to give the above concentrations. The samples were treated as described in Section 2.4 and analyzed by HPLC. The extraction efficiency was determined by comparing the peak areas of ibuprofen or (*S*)-naproxen to those of ibuprofen or (*S*)-naproxen solutions of corresponding concentration injected directly in the HPLC system without extraction.

2.7. Freeze–thaw stability of ibuprofen samples

The freeze–thaw stability of ibuprofen enantiomers was evaluated at four concentrations 0.1, 0.5, 5 and 50 μg/ml, using QC samples. These samples were analyzed in triplicate without being frozen at first, and then stored at −70 °C and thawed at room temperature (25 ± 1 °C) for three cycles.

The stability of ibuprofen in reconstituted extracts during run-time in the HPLC auto-injector was investigated using pooled extracts from QC samples of four concentration levels 0.1, 0.5, 5 and 50 μg/ml. Samples were kept in the sample rack of the auto-injector and injected into HPLC system every 4 h, from 0 to 24 h at the temperature of auto-injector (26 ± 1 °C).

2.8. Preliminary pharmacokinetic study in rats

Male Sprague–Dawley rats (350–450 g) were anaesthetized using halothane and a silastic catheter was cannulated into the right jugular vein. Animals were placed in metabolic cages, allowed to recover overnight and fasted for 12 h before dosing. On the day of experiments, animals were dosed orally with ibuprofen (25 mg/kg) suspended in 2% methycellulose. Serial blood samples (0.25 ml) were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h. After each sample collection, the cannula was flushed with 0.25 ml of saline. Following centrifugation of the blood samples, serum was collected and stored at −70 °C until analyzed.

2.9. Data analysis

Quantification was based on calibration curves constructed using peak area ratio (PAR) of ibuprofen to internal standard, against ibuprofen concentrations using unweighted least squares linear regression. Pharmacokinetic parameters were estimated using WinNonlin (version 1.0).

3. Results and discussion

3.1. Chromatography

Enantiomeric resolution of ibuprofen and baseline separation from the internal standard in rat serum was achieved using the ChiralPak AD-RH column. There were no interfering peaks co-eluted with the compounds of interest (Fig. 2A and B). The order of elution was determined by injecting a methanolic solution of (*S*)-ibuprofen under the current HPLC

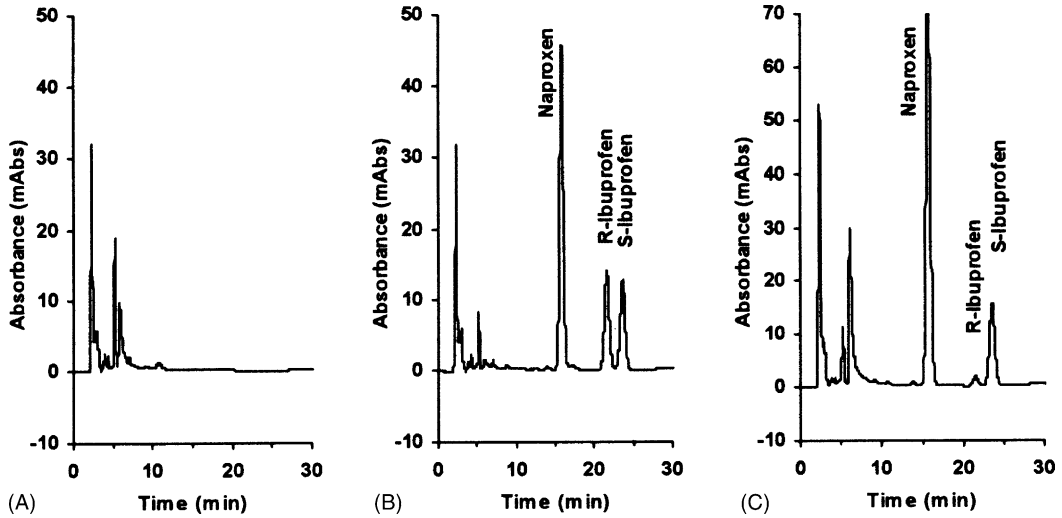

Fig. 2. Typical chiral-phase chromatograms of extracts of: (A) drug-free rat serum; (B) serum standard (enantiomers concentrations, 5 μg/ml) and the internal standard (*S*)-naproxen; (C) 4 h serum sample following oral administration of racemic ibuprofen to a rat (25 mg/kg).

conditions. The retention times of (*R*)- and (*S*)-ibuprofen were approximately 21.4 and 23.4 min, respectively (Fig. 2B). The internal standard was eluted at approximately 15.6 min. An additional small peak observed at 14 min (Fig. 2B and C) could possibly represent (*R*)-naproxen.

The performance of the HPLC assay was assessed using the following parameters, namely, interference from endogenous substances in rat serum, linearity, limit of quantitation (LOQ), freeze–thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. Various percentages of water, acetonitrile, phosphoric acid and triethylamine in the mobile phase were tested to achieve the best resolution between ibuprofen enantiomers. Replacement of acetonitrile with methanol resulted in no resolution between ibuprofen enantiomers. Several other chiral NSAIDs were also tested for separation on this column including flurbiprofen, ketorolac, etodolac, indoprofen, tiaprofenic acid, cicloprofen and ketoprofen. These tested chiral NSAIDs were totally or partially resolved except for etodolac and ketoprofen which were not resolved using a similar mobile phase to the one utilized in this assay (Table 1).

Table 1
Resolution and retention times of some chiral NSAIDs using the ChiralPak AD-RH column and a mobile phase of acetonitrile/0.08% phosphoric acid (4:6, v/v)

NSAID	Retention time (min)	Resolution (%)
Ketoprofen	6.5	No
Ketorolac	5.7, 8.2	100
Indoprofen	11.4, 12.6	80
Flurbiprofen	13.5, 17.0	100
Tiaprofenic acid	9.6, 10.4	80
Cicloprofen[a]	38.1, 45.8	100
Etodolac	6.1	No

[a] Cicloprofen was tested under the conditions for ibuprofen.

3.2. Linearity and LOQ

Excellent linear relationships ($r^2 = 0.9994$) were demonstrated between PAR of (*R*)- and (*S*)-ibuprofen to the internal standard and the corresponding serum concentrations over a range of 0.1–50 μg/ml for both ibuprofen enantiomers. The mean regression lines from the validation days were described by (*R*)-ibuprofen (μg/ml) = 10.07(PAR) − 0.0088, and (*S*)-ibuprofen (μg/ml) = 10.05(PAR) − 0.0016. The standard errors of the slopes of the calibration curves were 0.16 and 0.18 for (*R*)- and (*S*)-ibuprofen, respectively. The standard errors of the intercepts of the calibration curves were 0.03 and 0.04 for (*R*)- and (*S*)-ibuprofen, respectively. The LOQ of this assay

Table 2
Within- and between-day precision and accuracy of the serum assay for the enantiomers of ibuprofen (IB) after analysis of QC samples ($n = 6$)

Enantiomer concentration added (μg/ml)	Within-day						Between-day					
	Observed (μg/ml)		R.S.D. (%)		Bias (%)		Observed (μg/ml)		R.S.D. (%)		Bias (%)	
	(*R*)-IB	(*S*)-IB	(*R*)-IB	(*S*)-IB	(*R*)-IB	(*S*)-IB	(*R*)-IB	(*S*)-IB	(*R*)-IB	(*S*)-IB	(*R*)-IB	(*S*)-IB
0.10	0.11	0.11	10.40	10.32	12.15	14.80	0.11	0.11	4.05	4.86	9.13	10.35
0.25	0.23	0.24	5.31	4.76	−6.08	−2.18	0.24	0.25	6.49	7.69	−2.51	−1.87
5.00	5.00	5.07	7.49	7.38	0.01	1.42	5.00	5.02	0.64	1.21	0.02	0.48
50.00	50.61	49.91	6.54	3.49	1.23	0.19	50.88	50.26	1.79	1.33	1.77	0.52

Table 3
Recovery of ibuprofen enantiomers from rat serum ($n = 6$)

Enantiomer concentration (μg/ml)	Recovery (mean ± S.D., %)	
	(*R*)-Ibuprofen	(*S*)-Ibuprofen
0.1	85.2 ± 7.2	85.5 ± 7.4
0.5	87.6 ± 3.0	91.3 ± 3.1
5	99.1 ± 3.3	98.8 ± 3.1
50	94.5 ± 3.5	94.4 ± 3.3

was 0.1 μg/ml in rat serum with the corresponding relative standard deviation of 3.1 and 12.8% for (*R*)- and (*S*)-ibuprofen, respectively, and bias of 2.3 and 13.6% for (*R*)- and (*S*)-ibuprofen, respectively.

3.3. Precision, accuracy and recovery

The within- and between-day precision (R.S.D.) calculated following replicate analysis ($n = 6$) of ibuprofen enantiomers in rat serum was <10.32% over a wide range of concentrations (Table 2). The intra- and inter-day bias assessed during the replicate analyses for ibuprofen enantiomers varied between −6.1 and 14.8% (Table 2). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for ibuprofen enantiomers from rat serum varied from 85.2 to 99.1% (Table 3). In addition, the recovery of (*S*)-naproxen was 95.4% at its concentration used in the assay. High recovery from rat serum suggested that there was negligible loss of ibuprofen enantiomers and (*S*)-naproxen during the protein precipitation process. Additionally the efficiencies of extraction of ibuprofen enantiomers and (*S*)-naproxen were comparable.

3.4. Stability of ibuprofen samples

No significant degradation was detected after the samples of racemic ibuprofen in rat serum following three freeze–thaw circles for ibuprofen QC samples of 0.1, 0.5, 5 and 50 μg/ml. There was no significant decomposition observed after the reconstituted extracts of racemic ibuprofen were stored in the auto-injector at room temperature for 24 h.

3.5. Pharmacokinetics in rats

The HPLC method has been applied to the determination of ibuprofen enantiomers in the pharmacokinetic study in rats. Following oral administration of 25 mg/kg racemic ibuprofen, a rapid absorption and stereoselective disposition was observed for the enantiomers (Fig. 3 and Table 4). A typical chromatogram at 4 h post-dose illustrates dramatic differences in the concentrations at 4 h post-dose (Fig. 2C). Ibuprofen has previously demonstrated to have stereoselectivity in its pharmacokinetics in animals and humans [1].

Table 4
Mean pharmacokinetic indices after oral administration of 25 mg/kg of racemic ibuprofen to the rats (mean ± S.D., $n = 4$)

	C_{max} (μg/ml)	T_{max} (h)	$T_{1/2}$ (h)	AUC (μg h/ml)
(*R*)-Ibuprofen	9.54 (4.91)	0.25 (0.24)	2.3 (0.34)	29.9 (7.6)
(*S*)-Ibuprofen	24.1 (3.1)	0.5 (0.27)	2.1 (0.14)	97.9 (8.7)

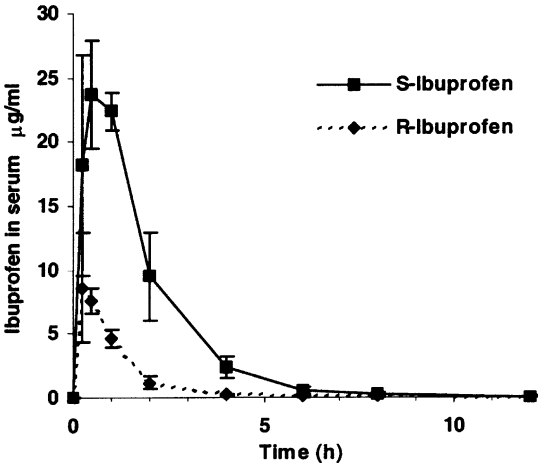

Fig. 3. Serum concentration-time profile of ibuprofen enantiomers following the oral administration of a suspension of racemic ibuprofen to rats (25 mg/kg; mean ± S.D., $n = 4$).

In summary, the developed HPLC assay is stereospecific, reproducible and accurate and sensitive. It has been successfully applied to the study of pharmacokinetics of this drug in rats. The present method offers significant advantages over those previously reported since many of these require lengthy pre-column derivatization times and result in co-extraction of significant amounts of endogenous compounds, and employ non-commercially available internal standards often have poor sensitivity and lengthy analysis times [5–9]. Using the present method, large numbers of biological samples may be analyzed in a relatively short period of time.

There are a large number of analytical methods using HPLC for separation and quantification of ibuprofen enantiomers. Derivatization with a chiral reagent may introduce inaccuracies into the determination of enantiomeric ration due to chiral impurities in the derivatization procedure and racemization during the derivatization procedure [10].

There are only a few direct methods of analysis of ibuprofen in plasma using chiral stationary phases [14–17]. In addition, the direct resolution of ibuprofen metabolites in urine on a ChiralPak AD-column in the normal phase mode has been previously validated [18]. The use of the chiral stationary phase β-cyclodextrin (Cyclobond I) was previously suggested to have a sensitivity of 0.1 μg/ml using a sample volume of 0.5 ml and is suitable for use in pharmacokinetic studies in humans and animals. However, this assay was validated without an internal standard [14].

A method based on α1-acid glycoprotein (AGP) bonded chiral stationary phase have been validated in chicken and human plasma. However, limit of quantification is only ∼1 μg/ml [16,17]. Another method using the AGP column Enantiopac® in human plasma also has a LOQ of 0.1 μg/ml using a 0.5 ml sample volume, however, this method does not achieve baseline resolution of the enantiomers [15].

As the AGP column is based on immobilized human plasma protein on silica particles, the separation power, decreases with an increasing number of plasma samples and may differ from one column to another. Thus, retention times, resolution and separation factors, and the quantitation limit may vary to a limited extent on the column used. Advantages of AGP are associated with the reverse-phase character of the column which allows direct injection of aqueous samples as does our assay. The sample volume used in the AGP assay is 0.5 ml and in our assay it is 0.1 ml. Therefore, at equivalent sample volumes our assay will be more sensitive with baseline resolution and for small animals where sample volumes are usually very limited we have demonstrated the utility of the present assay.

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