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Stereospecific high-performance liquid chromatographic analysis of flurbiprofen: application to pharmacokinetic studies

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Abstract

A method of analysis of flurbiprofen (+/− 2-(2-fluoro-4-biphenyl)-propionic acid) in biological fluids is necessary to study the kinetics of in vitro and in vivo metabolism and tissue distribution. A simple high-performance liquid chromatographic method was developed for simultaneous determination of flurbiprofen 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 (IS), *S*-naproxen and acidification with H_2SO_4. Separation was achieved on a Chiralpak AD-RH column with UV detection at 247 nm. The calibration curve was linear ranging from 0.05 to 50 μg/ml for each enantiomer. The mean extraction efficiency was >95%. Precision of the assay was <11% (CV), and was within 12.6% at the limit of quantitation (LOQ) (0.05 μg/ml). Bias of the assay was lower than 13.1%, and was within 12.8% at the LOQ. The assay was applied successfully to the in vivo kinetic study of flurbiprofen in rats.

Keywords: Reversed-phase HPLC; UV-detection; Kinetics, enantiomer, NSAID

1. Introduction

Flurbiprofen, +/− 2-(2-fluoro-4-biphenyl)-propionic acid, is a chiral 2-arylpropionic acid non-steroidal anti-inflammatory drug (NSAID) (Fig. 1). Flurbiprofen has been utilized since 1986 in the North American market and demonstrates stereoselectivity in its pharmacokinetics [1]. There is renewed interest in the use of NSAIDs including the enantiomers of flurbiprofen in the treatment of a variety of cancers including colorectal and prostate cancer [2–4].

Flurbiprofen has been previously stereochemically chromatographed utilizing a variety of indirect and direct methods [5]. There is only one completely validated previous direct method of stereospecific analysis of flurbiprofen in plasma using an α1-acid glycoprotein (AGP) column in reverse phase [6]. This method has good sensitivity of 0.05 mg/l using a sample volume of 0.5 ml. The Chiralpak AD-RH column is a newly commercially available tris(3,5-dimethylphenylcarbamate)

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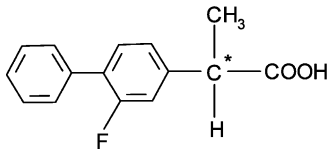

Fig. 1. Structures of flurbiprofen and its enantiomers.

of amylose which can be utilized in the reverse phase.

To our knowledge, no study has been published characterizing the separation of NSAID enantiomers using this column. The present study describes a stereoselective, isocratic, reversed-phase high performance liquid chromatography (HPLC) method for the determination of the enantiomers of flurbiprofen and possibly other NSAIDs and its application to in vivo kinetic studies.

2. Experimental

2.1. Chemicals and reagents

Racemic flurbiprofen and *S*-naproxen were purchased from Sigma Chemicals (St. Louis, MO, USA). HPLC grade acetonitrile, methanol, 2-propanol, and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). *S*-flurbiprofen, 2,2,4-trimethylpentane, sulphuric acid and phosphoric acid were from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). 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 diodearray 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 Chiralpak AD-RH column (150 × 4.6 mm I.D., 5-μm particle size, Chiral Technologies Inc. Exton, PA, USA). The mobile phase consisted of acetonitrile, water and phosphoric acid (40:60:0.05, 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 1 ml/min, with ultraviolet (UV) detection at 247 nm.

2.3. Stock and working standard solutions

A target amount of 25 mg of flurbiprofen 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 target concentration of 2 or 1 mg/ml. A methanolic stock solution of *S*-naproxen (internal standard, IS) was prepared similarly with the target concentration of 1 mg/ml. This solution was diluted with methanol to make a working IS solution of 100 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 flurbiprofen by sequential dilution with blank rat serum, yielding a series of concentrations namely, 0.1, 0.3, 1.0, 3.0, 10.0 and 100.0 μg/ml, in three replicates.

Quality control (QC) samples were prepared from the stock solution of flurbiprofen by dilution with blank rat serum to yield target concentrations of 0.1, 1.0, 10.0 and 100.0 μg/ml. The QC samples were divided into 0.1 ml aliquots in micro centrifuge tubes and stored at −70 °C before use.

2.4. Sample preparation

To the working standards or samples (0.1 ml) were added 25 μl of IS solution (100 μg/ml), 40 μl of 0.6 M sulphuric acid, and 2 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 2500 rpm for 5 min (Beckman J-6B centrifuge, Beckman Coulter, Inc., Fullerton, CA, USA 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 200 μl

of 70% methanol (v/v), vortexed for 1 min and centrifuged at 8000 rpm at 4 °C for 5 min, and 100 μl of the supernatant was injected onto the column.

2.5. Precision and accuracy

The within-run precision and accuracy of the replicate assays ($n = 6$) were tested by using four different concentrations of flurbiprofen enantiomers, namely 0.05, 0.5, 5 and 50 μg/ml. The between-run precision and accuracy of the assays were estimated from the results of six replicate assays of QC samples 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 [7].

2.6. Recovery

Recovery for flurbiprofen enantiomers from rat serum was assessed ($n = 6$) at 0.05, 0.5, 5 and 50 μg/ml and the recovery of the IS naproxen was evaluated at the concentration used in sample analysis (25 μg/ml). A known amount of flurbiprofen or naproxen was spiked into 0.1 ml rat serum to give the above concentrations. The samples were treated as described under Section 2.4 and analyzed by HPLC. The extraction efficiency was determined by comparing the peak areas of flurbiprofen or naproxen to those of flurbiprofen or naproxen solutions of corresponding concentration injected directly in the HPLC system without extraction.

2.7. Freeze–thaw stability of flurbiprofen samples

The freeze–thaw stability of flurbiprofen enantiomers was evaluated at four concentrations 0.05, 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 flurbiprofen in reconstituted extracts during run-time in the HPLC auto-injector was investigated using pooled extracts from QC samples of four concentration levels 0.05, 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. Pharmacokinetics of flurbiprofen in rat

Male Sprague Dawley rats (200–300 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 flurbiprofen (10 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, 12, 24, 28, 32, 36 and 48 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 flurbiprofen to IS, against flurbiprofen concentrations using unweighted least squares linear regression. Pharmacokinetic parameters were estimated using WinNonlin (version 1.0).

3. Results and discussion

3.1. Chromatography

Separation of flurbiprofen enantiomers and the IS in rat serum were achieved successfully. 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*-flurbiprofen under the current HPLC conditions. The retention times of *R*- and *S*-flurbiprofen were approximately 12 and 16 min, respectively (Fig. 2B). The IS was evident at approximately 9 min.

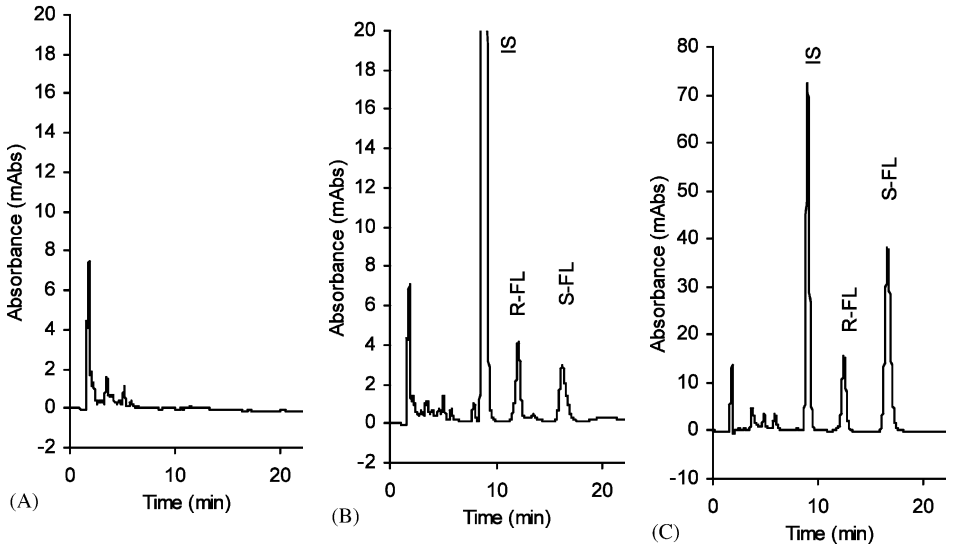

Fig. 2. Representative chromatograms, of (A) drug-free rat serum, (B) serum containing flurbiprofen (FL) enantiomers each with concentration of 0.5 μg/ml and the IS, and (C) 4 h serum sample containing flurbiprofen enantiomers and the IS.

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in rat serum, linearity, limit of quantitation (LOQ), freeze–thaw stability, stability of reconstituted extracts, precision, accuracy and recovery. Various composition of mobile phase was tested to achieve the best resolution between flurbiprofen enantiomers. Replacement of acetonitrile with methanol resulted in no separation between flurbiprofen enantiomers. Several other chiral NSAIDs were also tested for separation on this column including ibuprofen, ketorolac, etodolac, indoprofen, tiaprofenic acid 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. Hence, other chrial NSAIDs may also be suitably chromatographed using this type of chiral column (Table 3).

The other validated stereospecific assay of flurbiprofen using an AGP column has a similar sensitivity to the current assay of 50 ng/ml, however, larger sample volumes of 0.5 ml of plasma were necessary [6]. The present assay is more practical to use in pre-clinical applications of flurbiprofen where small sample volumes are obtained. 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, separation factors, and the quantitation limit may vary to a limited extent on the column used.

3.2. Linearity, LOQ and LOD

Excellent linear relationships ($r^2 = 0.9999$) were demonstrated between PAR of *R*- and *S*-flurbiprofen to the IS and the corresponding serum concentrations of flurbiprofen enantiomers over a range of 0.05 to 50 μg/ml. The mean regression lines from the validation runs was described by *R*-flurbiprofen (μg/ml) = PAR × 11.34 − 0.007 and *S*-flurbiprofen (μg/ml) = PAR × 11.38 − 0.004. The LOQ of this assay was 0.05 μg/ml in rat serum with the corresponding R.S.D. of 10 and 10.7% for *R*- and *S*-flurbiprofen, respectively, and bias of 4 and 12.8% for *R*- and *S*-flurbiprofen, respectively. The back-calculated concentration of QC samples was within the acceptance criteria.

3.3. Precision, accuracy and recovery

The within- and between-run precision (R.S.D.) calculated during replicate assays ($n = 6$) of flurbiprofen enantiomers in rat serum was $< 12.6\%$ over a wide range of concentrations (Table 1). The intra- and inter-run bias assessed during the replicate assays for flurbiprofen enantiomers varied between -3.4 and 13.1% (Table 1). These data indicated that the developed HPLC method is reproducible and accurate. The mean extraction efficiency for flurbiprofen enantiomers from rat serum varied from 96.0 to 100.3% (Table 2). In addition, the recovery of *S*-naproxen was 98.1% at its concentration used in the assay. High recovery from rat serum suggested that there was negligible loss of flurbiprofen enantiomers and *S*-naproxen during the protein precipitation process. Additionally the efficiencies of extraction of flurbiprofen enantiomers and *S*-naproxen were comparable.

Table 2
Recovery of flurbiprofen enantiomers from rat serum ($n = 6$)

Concentration (μg/ml)	Recovery% (Mean ± S.D.)	
	R-flurbiprofen	*S*-flurbiprofen
0.05	96.0 ± 5.4	99.5 ± 9.9
0.5	100.2 ± 5.1	100.0 ± 5.0
5	97.6 ± 2.2	100.3 ± 1.7
50	99.9 ± 5.3	100.3 ± 5.0

Table 3
Resolution and retention times of chiral NSAIDs using chiral Pak AD-RH column

NSAID	Retention time (min)	Resolution
Ketoprofen	6.5	No
Ketorolac	5.7, 8.2	100%
Indoprofen	11.4, 12.6	80%
Ibuprofen	11.5, 12.7	80%
Tiaprofenic acid	9.6, 10.4	80%
Etodolac	6.1	No

3.4. Stability of flurbiprofen samples

No significant degradation was detected after the samples of racemic flurbiprofen in rat serum following three freeze–thaw circles. The recoveries of *R*- and *S*-flurbiprofen were, respectively, from 91.5 to 106.8 and 96.1 to 106.9% following three freeze–thaw cycles for flurbiprofen QC samples of 0.05, 0.5, 5 and 50 μg/ml. There was no significant decomposition observed after the reconstituted extracts of racemic flurbiprofen were stored in the auto-injector at room temperature for 24 h. The measurements were from 97.1 to 112.1% of the initial value for extracts of racemic flurbiprofen in rat serum of 0.05, 0.5, 5 and 50 μg/ml, respectively, during the storage in the auto injector at room temperature for 24 h.

3.5. Pharmacokinetics of flurbiprofen in rats

The HPLC method has been applied to the determination of flurbiprofen enantiomers in the

Table 1
Within- and between-run precision and accuracy of the assay for flurbiprofen (FL) enantiomers in rat serum

Enantiomer concentration (μg/ml)					R.S.D.%				Bias%			
Added	Observed				Within-run		Between-run		Within-run		Between-run	
	Within-run		Between-run		*R*-FL	*S*-FL	*R*-FL	*S*-FL	*R*-FL	*S*-FL	*R*-FL	*S*-FL
	R-FL	*S*-FL	*R*-FL	*S*-FL								
0.05	0.052	0.056	0.049	0.048	9.2	8.7	12.6	10.4	4.0	12.8	−2.8	−3.4
0.5	0.57	0.53	0.55	0.51	3.3	4.1	3.7	4.4	13.1	6.2	10.4	2.4
5	4.93	4.96	4.95	4.99	2.9	1.2	0.5	0.5	−1.5	−0.8	−1.0	−0.2
50	50.0	50.0	50.0	50.0	1.9	2.4	0.0	0.0	0.0	0.0	0.0	0.0

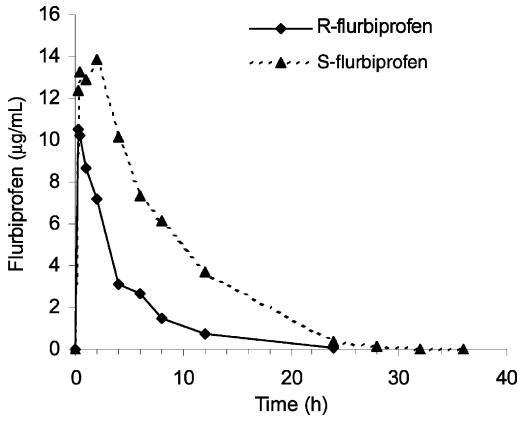

Fig. 3. Concentration–time profile of flurbiprofen enantiomers following the administration of a suspension of flurbiprofen orally to a rat (10 mg/kg).

pharmacokinetic study in rats. Following oral administration of racemic flurbiprofen, a rapid absorption and stereoselective disposition was observed for the enantiomers (Fig. 3). Flurbiprofen has previously demonstrated to have stereoselectivity in its pharmacokinetics [8,9].

In summary, the developed HPLC assay is stereospecific, reproducible and accurate. It has been successfully applied to the study of pharmacokinetics of this drug in rats.

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