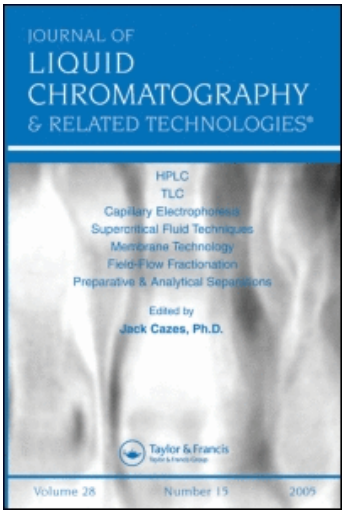

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Determination of Ibuprofen Enantiomers in Human Plasma by Derivatization and High Performance Liquid Chromatography with Fluorescence Detection

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DETERMINATION OF IBUPROFEN ENANTIOMERS IN HUMAN PLASMA BY DERIVATIZATION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A sensitive and selective reverse phase high performance liquid chromatographic (HPLC) method was developed for the determination of the enantiomers of ibuprofen in human plasma. Ibuprofen and fenoprofen (internal standard, ISTD) are extracted from human plasma by n-butyl chloride, following acidification of plasma. The method is based on the separation of the diasteromers formed on reacting ibuprofen enantiomers and ISTD with S-(-)-1-(1-naphthyl)ethylamine. Separation is achieved by HPLC on an Inertsil ODS-2 column,with a mobile phase composed of water (pH 3.0):ACN (33.5:66.5). Detection is by fluorescence detection with excitation and emission at 280 and 320 nm, respectively. The mean retention times of S-(+)-ibuprofen, R-(-) -ibuprofen, S-fenoprofen and R-fenoprofen (ISTD) are approximately 11.3, 12.3, 7.7 and 8.5 minutes, respectively.

The assay is linear in concentration ranges of 100 to 20,000 ng/mL. The analysis of pooled quality controls (400, 2,000, and 16,000 ng/mL) demonstrates excellent precision with relative standard deviations (RSD) (n=18) range from 3.6% to 8.8%. The method is accurate with all intraday (n=6) and overall (n=18) mean values for the quality control samples being less than 7.0% from theoretical.

INTRODUCTION

Ibuprofen (IB, (±)-2-(4-isobutylphenyl)propionic acid), is an effective and well tolerated 2-arylpropionic acid non-steroidal anti-inflammatory drug. It contains a chiral center and is marketed as a 50:50 mixture of the S(+)- and R-(-)-enantiomers, although the pharmacological activities of ibuprofen are mainly associated with the S-(+)-enantiomer.[1-3] It is necessary to develop methods to quantitate the enantiomers of ibuprofen.

To date, there have been numerous analytical methods developed using GC or HPLC for the separation and quantitation of IB enantiomers in biological specimens.[4-22] However, these methods suffered lengthy sample preparation, presence of endogenous interferences, poor sensitivity, expensive chiral stationary phases and extensive column flushing procedure which, result in lengthy analysis time.

In this manuscript, a simple, sensitive and specific method is described for the determination of ibuprofen enantiomers in human plasma. The assay is based on the separation of the diasteromers formed on reacting ibuprofen enantiomers and ISTD with S-(-)-1-(1-naphthyl)ethylamine and chloroformate as the coupling reagent.

EXPERIMENTAL

Materials

R-(-)- and S-(+)-ibuprofen were obtained from RBI (Natick, MA, USA). Fenoprofen calcium (FENO, internal standard) was obtained from Sigma (St. Louis, MO, USA). Heparinized human plasma was obtained from Valley Biomedical. Acetonitrile, and n-Butyl chloride, HPLC grade were obtained from Burdick & Jackson (Muskegon, MI, USA). Acetic acid, and Sulfuric acid, GR grade, were obtained from EM Science (Gibbstown, NJ, USA). Ethyl chloroformate, ethanolamine and S-(-)-1-(1-naphthyl)ethylamine were obtained from Aldrich (Milwaukee, WI, USA).

Triethylamine was obtained from Sigma (St. Louis, MO, USA). Deionized water was processed through a Milli-Q water purification system, Millipore Corporation.

Chromatographic Systems

The HPLC system consisted of a Perkin-Elmer 200 LC pump (Norwalk, CT, USA), a Waters 717 autoinjector (Milford, MA, USA), and Jasco FP-920 fluorescence detector (Tokyo, Japan), with excitation and emission wavelength at 280 and 320 nm, respectively. The analytical column was an Alltech Inertsil ODS-2, 150 mm x 4.6 mm, 5-μm particle size (Deerfield, IL, USA), protected by a Brownlee Newguard RP-18 pre-column (15 mm x 3.2 mm, 7-μm particle size, ABI, San Jose, CA, USA). Data collection and calculations were conducted with an HP1000 Model A990 computer with a 3350A Laboratory Automation System (Hewlett-Packard, Palo Alto, PA, USA). The mobile phase was water (pH 3.0)/acetonitrile (33.5:66.5) with a flow rate of 1.2 mL/min. The column was maintained at 27°C with an Eppendorf CH-30 column heater (Madison, WI, USA).

Preparation of Standard Solutions

Stock standard solutions of R-(-)- and S-(+)-ibuprofen (1 mg/mL) were prepared by dissolving 25 mg of R-(-)- or S-(+)-ibuprofen in 25 mL of methanol. A stock solution of internal standard (100 μg/mL) was prepared by dissolving 2.5 mg of fenoprofen in 25 mL of methanol. Working solutions of R-(-)- and S-(+)-ibuprofen (1 to 200 μg/mL) were prepared by diluting the stock solution with 25:75 methanol/water. The internal standard working solution (50 μg/mL) was prepared by diluting the stock solution with water. The R-(-)- and S-(+)-ibuprofen and the internal standard solutions were stored at 4°C. All solutions were stable for at least 1 month.

Quality Control Samples

Pooled quality control samples (QC samples) were prepared to determine the precision and accuracy of the method, and to evaluate the stability of samples. A control pool was also prepared at a concentration above the curve range (over-cure control) to evaluate precision and accuracy when specimens required analysis at partial volume.

Plasma control pools (400, 2000 and 16000 ng/mL) were prepared by diluting 100 μL of 100 μg/mL, 500 μL of 100 μg/mL, and 400 μL of 1 mg/mL R-(-)- and S-(+)-ibuprofen, respectively, to a 25-mL volume, using blank human

plasma. An over-curve control (30000 ng/mL) was prepared by diluting 300 μL of 1 mg/mL to a 10-mL volume with blank human plasma. All control pools were aliquoted into 4-mL polypropylene-vials and stored at approximately -20°C.

Sample Preparation

Calibration standards were prepared by adding 50 μL of R-(-)- and S-(+)-ibuprofen working solutions to 0.5 mL of blank human plasma. Clinical specimens and controls were prepared by aliquoting 0.5 mL of plasma into glass tubes.

Calibration standards, clinical specimens and controls were processed by adding 50 μL of internal standard, 200 μL of 20% sulfuric acid and 6 mL of n-butyl chloride. The samples were mixed on a vortex mixer for 5 minutes, and centrifuged at approximately 950 g for 5 minutes. The aqueous layer was frozen in a dry ice-acetone bath, the organic layer was transferred to a clean tube. The n-butyl chloride was evaporated to dryness under nitrogen.

Derivatization Procedures

The samples were reconstituted in 300 μL of 50 mM TEA, sonicated for 1 minute and mixed on a Vortex mixer for 30 s. Fifty microliters of 6 mM ethyl chloroformate was added to each tube and after 30 s, 25 μL of 10 mM S-(-)-1-(1-naphthyl)ethylamine was added. Three minutes later 25 μL of 1:40 ethanolamine/acetonitrile was added. The solvent was evaporated under nitrogen in TurboVap at 40°C and reconstituted in 250 μL of mobile phase. Twenty five microliters aliquots were injected into the HPLC system.

Validation

Duplicate calibration curves were analyzed on each of the three days of validation. One reagent blank (water substituted for plasma), blank plasma, control zero (blank plasma spiked with internal standard) and triplicate controls at each concentration (400, 2000 and 16000 ng/mL of R-(-)- and S-(+)-ibuprofen in plasma) were analyzed with each calibration curve. The calibration curves were obtained by weighted (1/C) least-squares linear regression analysis of the peak height ratios of R-(-)- or S-(+)-ibuprofen/internal standard vs. the concentration of R-(-)- or S-(+)-ibuprofen, respectively. The equations of the calibration curves were then used to calculate the concentration of R-(-)- or S-(+)-ibuprofen in the samples and controls from their peak height ratios.

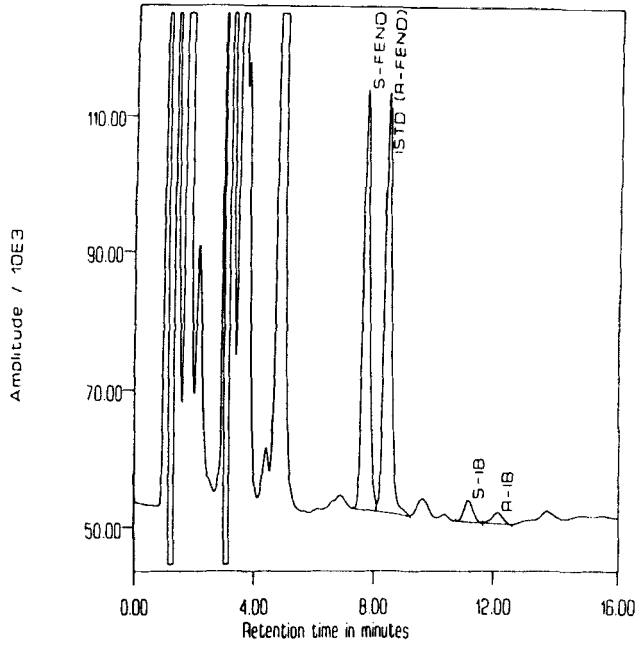

Figure 1. Chromatogram of a 100-ng/mL Calibration Standard.

RESULTS AND DISCUSSION

The diasteriomers of ibuprofen and fenoprofen were well separated from each other, as shown in representative chromatograms (Figures 1 - 3). The retention times of S-(+)-ibuprofen, R-(-)-ibuprofen, S-fenoprofen and R-fenoprofen (ISTD) were approximately 11.3, 12.3, 7.7 and 8.5 minutes, respectively.

Blank human plasma from twelve pools was tested for endogenous interferences. No endogenous interferences were found in the ibuprofen and ISTD regions.

Precision, and Accuracy of S-(+)-ibuprofen, and R-(-)-ibuprofen standards are in Tables 1 and 2. The standards show low values in deviation (<6.1%) and relative standard deviation (<5.9%). Calibration curves for S-(+)-ibuprofen, and R-(-)-ibuprofen in plasma were linear over the concentration range of 100 to 20000 ng/mL, with correlation coefficients greater than 0.9997 for both enantiomers.

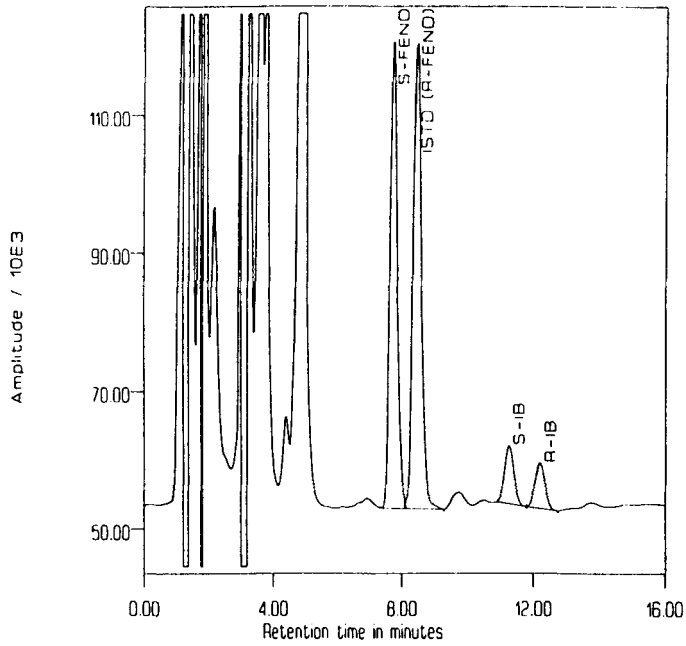

Figure 2. Chromatogram of an 400-ng/mL Quality Control Sample.

Data from the quality control samples are shown in Table 3 and 4. The within-day precision of the method as measured by the RSD of the daily mean (n = 6), ranged from 2.1% to 11.3% at the three control concentrations in human plasma. The overall precision ranged from 3.6% to 8.8% RSD (n = 18) for the 400-, 2000- and 16000-ng/mL of the S-(+)- and R-(-)-ibuprofen controls.

The accuracy of the method was determined by comparing the means of the measured concentrations with the nominal (theoretical) concentrations of S-(+)-and R-(-)-ibuprofen in the plasma controls. All of the daily mean (n = 6) and overall mean (n = 18) values for the controls were within 7.0% of their expected values.

A QC sample pool containing 30000 ng/mL of S-(+)-ibuprofen and R-(-)-ibuprofen was prepared and analyzed with the high QC sample (16000 ng/mL) at the partial volumes of 50 μL. These aliquots were diluted to a final volume of 500 μL with blank plasma. The mean (n = 6) values for all partial volumes were within 6.9% of their expected values. The precision was better than 4.7% RSD (n = 6) at all partial volumes.

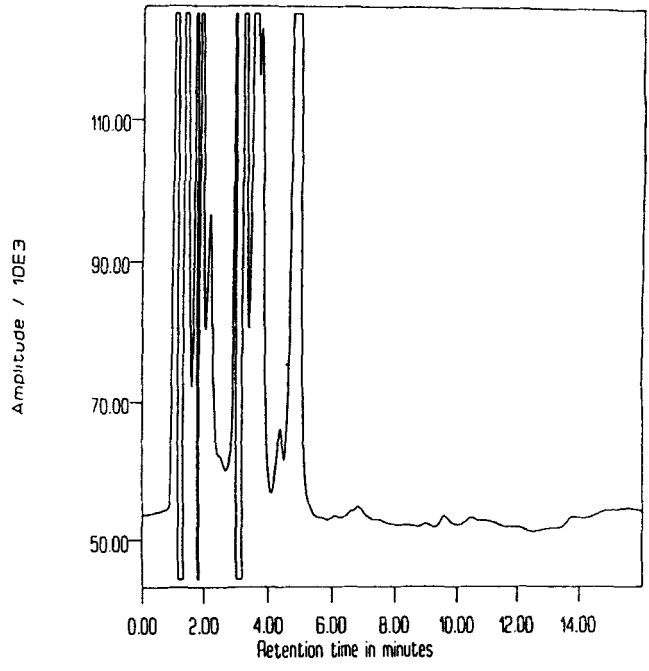

Figure 3. Chromatogram of Blank Human Plasma.

Table 1

Precision and Accuracy of S-(+)-Ibuprefen Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (mean ± S.D., n=6) (ng/mL)	R.S.D. (%)	Deviation (%)
100	101 ± 2.3	2.3	1.4
200	202 ± 6.96	3.4	1.1
500	502 ± 23.9	4.8	0.5
1000	987 ± 20.9	2.1	-1.4
5000	4910 ± 97.5	2.0	-1.8
10000	9950 ± 134	1.3	-0.5
20000	20200 ± 140	0.7	1.0

Table 2

Precision and Accuracy of R-(+)-Ibuprefen Standards

Calibration Standard Concentration (ng/mL)	Calculated Concentration (mean ± S.D., n=6) (ng/mL)	R.S.D. (%)	Deviation (%)
100	106 ± 4.6	4.4	6.1
200	199 ± 11.7	5.9	-0.3
500	489 ± 14.5	3.0	-2.1
1000	980 ± 35.0	3.6	-2.1
5000	4910 ± 115	2.4	-1.8
10000	9950 ± 138	1.4	-0.5
20000	20200 ± 190	0.9	1.0

Table 3

Precision and Accuracy of S-(+)-Ibuprofen Quality Controls

Control Concentration (ng/mL)	Calculated Concentration (Overall mean ± S.D., n-=18) (ng/mL)	R.S.D (%)	Deviation (%)
400	407 ± 17.3	4.3	1.6
2000	1960 ± 166	8.5	-1.9
16000	15600 ± 580	3.7	-2.6

Table 4

Precision and Accuracy of R-(+)-Ibuprofen Quality Controls

Control Concentration (ng/mL)	Calculated Concentration (Overall mean ± S.D., n-=18) (ng/mL)	R.S.D (%)	Deviation (%)
400	395 ± 14.2	3.6	-1.6
2000	1950 ± 173	8.8	-2.5
16000	15800 ± 585	3.7	-1.2

The lower limit of quantitation (LLOQ) was set at 100 ng/mL of S-(+)-, and R-(-)-ibuprofen in human plasma. Six replicates of the lowest standard (100 ng/mL) were analyzed to evaluate the accuracy and precision at the LLOQ. At the LLOQ, the RSD (n = 6) of the peak height ratios was 10.2%, the RSD (n = 6) of the measured concentrations was 14.0%, and the deviation of the mean (n=6) of the measured concentrations from their nominal value was 7.0%.

Extraction recoveries were determined by comparing the peak heights of extracted calibration standards with the peak heights of pure recovery standards at the same nominal concentrations. The mean recoveries for S-(+)-ibuprofen, R-(-)-ibuprofen and the internal standard were 76.6%, 76.1%, and 89.6%, respectively.

Stability was tested by subjecting the QC samples to three freeze/thaw cycles, and storage for 24 hours at room temperature. The thawing and refreezing of QC samples and the storage of QC samples at room temperature, had little effect on the precision or accuracy of the results. The mean (n = 3) value was within 7.5% of the expected values.

Process stability was tested by extracting one set of calibration standards with duplicate QC samples and stored overnight at room temperature before analyzing. The storage of extracted samples at room temperature had little effect on the accuracy and precision of the results. The mean (n = 2) values of the controls was within 9.0% of the expected values.

In conclusion, the method presented here for the determination of S-(+)-ibuprofen and R-(-)-ibuprofen enantiomers in human plasma is precise, and accurate. The ruggedness of the procedure has been demonstrated by applying the methods for the analysis of calibration standards and quality controls.

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