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Short Communication

Stereospecific high-performance liquid chromatographic assay of ibuprofen: improved sensitivity and sample processing efficiency

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ABSTRACT

A high-performance liquid chromatographic (HPLC) assay suitable for the analysis of the enantiomers of the non-steroidal anti-inflammatory drug ibuprofen (IB) in plasma was developed. Following the addition of racemic fenoprofen as internal standard (I.S.), samples are acidified and extracted with a mixture of isooctane–isopropanol (95:5, v/v). After evaporation of the organic layer, the drug and I.S. are derivatized with *S*-(−)-1(1-naphthyl)ethylamine (S-NEA) after addition of ethyl chloroformate as the coupling reagent. Ethanolamine is added 3 min after the addition of S-NEA to react with the excessive ethyl chloroformate. The resultant diastereomers corresponding to IB and I.S. were chromatographed at ambient temperature on a 100 mm × 4.6 mm I.D. C_{18} reversed-phase column using acetonitrile–water–acetic acid–triethylamine (60:40:0.1:0.02) as the mobile phase pumped at a flow-rate of 1.2 ml/min. Detection of the fluorescent chromophore was at 280 and 320 nm for excitation and emission, respectively. The suitability of the assay for clinical pharmacokinetic studies of IB was determined by the analysis of plasma samples obtained from a healthy volunteer, following administration of a single 400-mg oral dose of racemic IB.

INTRODUCTION

Ibuprofen [IB, (±)-2-(4-isobutylphenyl)propionic acid], a 2-arylpropionic acid (2-APA) non-steroidal anti-inflammatory drug (NSAID), contains a chiral center and is marketed as a 50:50 mixture of the *S*-(+)- and *R*-(−)-enantiomers. As with other drugs within this class of 2-APA NSAIDs, the *S*-(+) isomer is considered to have greater desired pharmacologic activity then the *R*-(−) isomer [1]. Consequently, as previously well documented by others [2–17], studies that

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necessitate the quantification of IB in biological samples must utilize stereospecific techniques.

To date, there have already been numerous reports of analytical techniques utilizing gas chromatography (GC) or high-performance liquid chromatography (HPLC) for the separation and quantitation of IB enantiomers in biological specimens (*e.g.* refs. 3–17). Of these methods, however, several limitations including lengthy sample preparation procedures, the presence of endogenous peaks co-eluting with the analyte of interest, limited assay sensitivity and relatively expensive chiral stationary phases have been reported.

In this article we report an HPLC method for the determination of IB enantiomers which utilizes fluorescence detection and solvent switching to offer enhanced sensitivity, reduced sample preparation time and reduced run times.

EXPERIMENTAL

Chemicals

Racemic IB and fenoprofen calcium [internal standard (I.S.) (±)-α-2-(3-phenoxyphenyl)propionic acid] were obtained from Aldrich (Milwaukee, WI, USA) and Eli Lilly and Company (Indianapolis, IN, USA), respectively. Ethyl chloroformate (ECF), ethanolamine (EOA) and *S*-(−)-1-(1-naphthyl)ethylamine (S-NEA) were also obtained from Aldrich. Analytical-grade acetic and sulphuric acid, HPLC-grade triethylamine (TEA) and chloroform were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Solvents including acetonitrile, methanol, water, isopropanol and isooctane were HPLC grade and were obtained from BDH (Toronto, Canada).

Chromatography

The HPLC apparatus consisted of a Model 590 programmable double-piston pump with a solvent select valve attached to a WISP Model 712 autosampler, a Model 470 scanning fluorescence detector, and a NEC Powermate 386SX Plus computer with Maxima 820 data acquisition software (Waters Scientific, Mississauga, Canada). Separation of the analytes was accomplished using a 5-μm reversed-phase Partisil ODS 3 RAC II column (10 cm × 4.6 mm I.D.) (Whatman, Clifton, NJ, USA). Fluorescence detection was at 280 and 320 nm for excitation and emission, respectively, with detector gain set at 100 times. The mobile phase, pumped at a flow-rate of 1.2 ml/min, consisted of acetonitrile–water–acetic acid–TEA (60:40:0.1:0.02, v/v). The final pH of the mobile phase was 5.0. After every third injection the system was programmed to flush using the solvent select valve with 100% acetonitrile for 6 min at a flow-rate of 1.6 ml/min; the system was allowed to equilibrate with mobile phase for 9 min prior to the next sample injection.

Standard solutions

To 20 ml of HPLC-grade methanol were added 20 mg of racemic IB which was then diluted to a total volume of 100 ml with HPLC-grade water. The I.S. solution consisted of 20 mg of racemic fenoprofen which was dissolved in 20 ml of HPLC-grade methanol and diluted to a total volume of 100 ml with HPLC-grade water. These solutions were stored at 5°C. To drug-free plasma was added IB to give final enantiomer concentrations of 0.1, 0.5, 1, 2, 10 and 20 μg/ml.

Solutions of TEA (50 mmol/l), ECF (6 mmol/l) and EOA (1:40, v/v) were prepared in acetonitrile. The S-NEA was prepared in a 2% solution of TEA in acetonitrile.

Sample preparation

To 0.5 ml of plasma containing IB were added 50 μl of I.S. solution and 200 μl of 1 *M* sulphuric acid. Samples were then extracted with 3 ml of a mixture of isooctane–isopropanol (95:5, v/v). The resultant mixture was vortex-mixed for 30 s using a Vortex Genie 2 mixer (Fisher Scientific, Edmonton, Canada) and centrifuged for 5 min at 1800 *g* using a Dynac II centrifuge (Becton Dickinson, Parsippany, NJ, USA). The organic phase was transferred to a clean tube and evaporated to dryness using a Savant Speed Vac concentrator–evaporator (Fisher Scientific, Edmonton, Canada). The remaining residue was reconstituted in 300 μl of 50 m*M* TEA in acetonitrile. To this 50 μl of 6 m*M* ECF in acetonitrile and, 30 s

later, 25 μl of the S-NEA solution were added. After allowing the mixture to react for 3 min, 25 μl of EOA–acetonitrile were added to stop the ECF from reacting further. Aliquots ranging from 2 to 30 μl were injected into the HPLC column. Both sample preparation and analysis were conducted at ambient temperature (22–25°C).

Quantitation

Calibration curves were constructed by plotting the peak-area ratios (IB/I.S.) obtained from the analysis of drug *versus* the corresponding enantiomer concentration added to plasma. The first elution peak of the I.S. was used for quantification of the peak-area ratio. Results are reported as mean ± S.D.

Accuracy and precision

Drug-free plasma was spiked with IB at six different enantiomer concentrations ($n = 9$ for each concentration) over the range 0.1–20.0 μg/ml. Accuracy was assessed by determining the concentration of drug measured in each sample relative to the known concentration added and was expressed as the analytical recovery (%). Precision was determined utilizing the coefficient of variation (%) of the inter-day variations of the standard curves.

RESULTS AND DISCUSSION

Fig. 1 depicts chromatograms of blank plasma, plasma samples spiked with 0.1 and 10.0 μg/ml of each IB enantiomer, and a 5-h plasma sample from a healthy subject dosed with a single 400-mg IB tablet. Peaks illustrating *S*-(+)- and *R*-(−)-IB eluted at approximately 10.5 and 11.8 min, respectively. Peaks corresponding to *S*-fenoprofen and *R*-fenoprofen (I.S.) were eluted at approximately 7.5 and 8.8 min, respectively. The order of elution has previously been reported by Mehvar *et al.* [10].

Blank plasma samples were free of any interfering peaks. Calibration curves for *S*-(+)- and *R*-(−)-IB were typically described by $y = -0.0647 + 1.1127x$ and $y = -0.0371 + 1.0295x$, where y is the enantiomer concentration and x the peak-area ratio. The calculated peak-area ratios and the added concentrations (0.1–20 μg/ml) displayed an excellent linear relationship with a correlation coefficient which was typically 0.999 for both enantiomers. The assay was accurate and precise as summarized in Table I. Precision, as determined by the coefficient of variation of the inter-day variations of the standard curves using the peak-area ratios, was consistently less than 10–15%. Accuracy, indicated by the analytical

TABLE I

ACCURACY AND PRECISION OF THE ASSAY

Concentration added (μg/ml)	Measured concentration (mean ± S.D.) (μg/ml)		Accuracy (analytical recovery, %)		Precision (C.V., %)	
	S-IB	*R*-IB	*S*-IB	*R*-IB	*S*-IB	*R*-IB
0.1	0.10 ± 0.01	0.10 ± 0.01	104	101	15.6	14.9
0.5	0.52 ± 0.05	0.51 ± 0.04	103	101	7.86	5.70
1.0	1.00 ± 0.04	1.00 ± 0.04	100	99.8	3.27	3.13
2.0	1.98 ± 0.06	2.00 ± 0.08	99.1	100	2.94	4.21
10.0	10.1 ± 0.24	10.1 ± 0.22	101	101	3.82	4.31
20.0	20.0 ± 0.12	20.0 ± 0.11	99.8	99.9	4.44	5.60

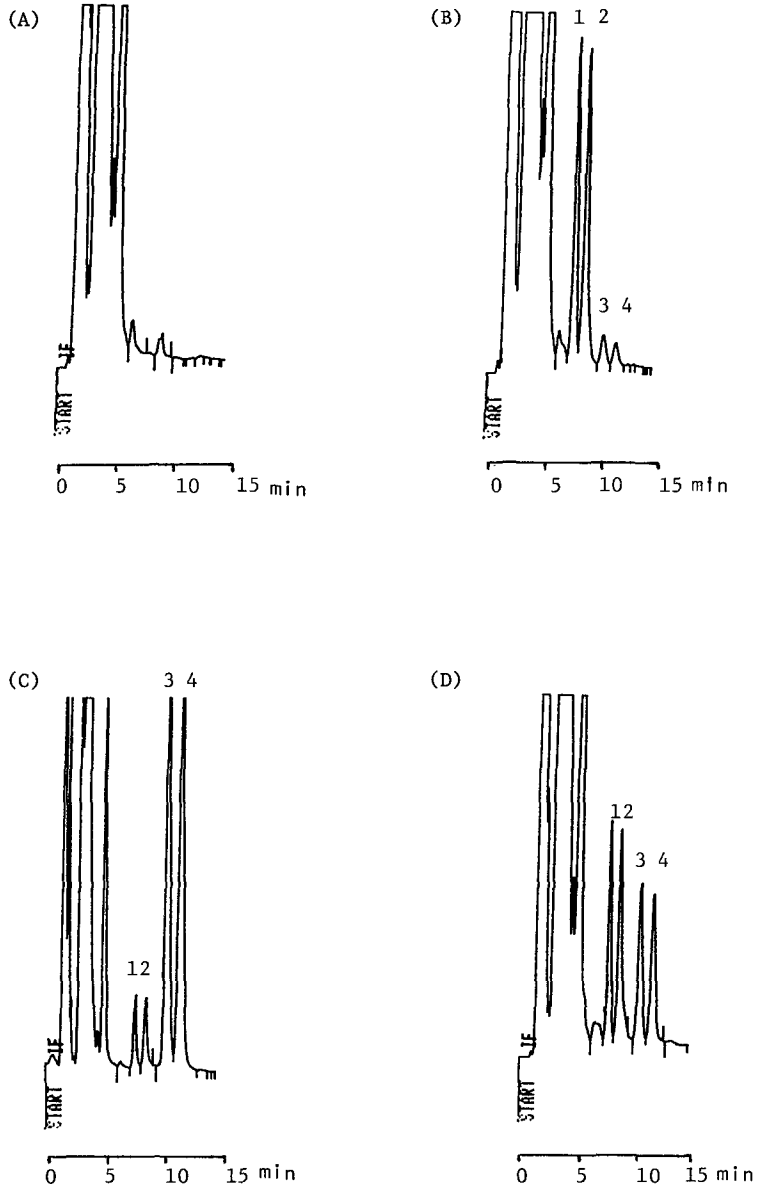

Fig. 1. Chromatograms of (A) blank plasma, (B) plasma spiked with 0.1 μg/ml of each IB enantiomer, (C) plasma spiked with 10.0 μg/ml of each IB enantiomer, and (D) plasma sample taken 5.0 h after an oral 400-mg dose of racemic IB; *R*- and *S*-IB corresponded to 4.4 and 5.7 μg/ml, respectively. Peaks: 1 = *S*-I.S.; 2 = *R*-I.S.; 3 = *S*-IB; 4 = *R*-IB.

recovery was calculated to be approximately 100% for both *S*-(+)- and *R*-(−)-IB throughout the concentration range examined. Although the lowest calibration concentration was 0.1 μg/ml, greater sensitivity in the order of 10 ng/ml was obtainable using the described chromatographic conditions. For the concentration range described in this report, injection volumes ranging from 2 to 30 μl of a total 400-μl volume per sample were used. Therefore, a 30-μl injection volume of a 0.1 μg/ml sample actually loaded only 7.5 ng of each enantiomer of IB onto the column. Consequently, by simply increasing the size of the injection volume, sensitivity could be enhanced if required.

The analysis of IB in biological samples has utilized a number of different GC and HPLC stereospecific techniques [3–17]. Of these techniques, enantiomer separation has been accomplished via pre-column derivatization of the IB with a homochiral reagent [3–6,10,11,15,16] or by direct injection of the enantiomers onto a chiral stationary phase [7–9,12–14,17]. The latter methods, however, require the use of rather expensive columns which may have a somewhat limited lifetime. For example, Pettersson *et al.* [8] separated and quantified IB enantiomers with an α_1-acid glycoprotein stationary phase. In their report, however, column efficiency decreased after only 100–150 injections. Furthermore, it was necessary to restore the column by flushing overnight with a 25% 2-propanol in water solution. Although pre-column derivatization of IB with a homochiral reagent has been reported by others, some limitations have included the need for two extraction steps [3,10], the presence of endogenous materials which co-extracted with IB [10], and lengthy derivatization steps [4–6,11,15, 16].

The present assay was adapted from that reported by Mehvar *et al.* [10], which utilized S-NEA as the derivatizing reagent with subsequent UV detection of the diastereomers corresponding to IB. However, by using a fluorescent detector to detect the naphthyl chromophore, sensitivity was enhanced with respect to the signal-to-noise ratio so that low nanogram quantities were detectable. Additionally, by changing the detector gain from 100 to 1000 times, even greater sensitivity than previously reported was obtainable, while maintaining an acceptable signal-to-noise ratio. The enhanced sensitivity compared with previous reports is advantageous if either the volume of the available plasma samples is limited, or if plasma samples are collected over an extended period of time following dosage administration. The increased sensitivity obtained using the fluorescence detector, however, also resulted in the detection of a late-eluting peak which necessitated run times in excess of 60 min under isocratic conditions. Consequently, a solvent select valve enabled the late-eluting peak to be rapidly flushed out after every third sample using 100% acetonitrile which was delivered at a flow-rate of 1.6 ml/min for 6 min. This necessitated re-equilibration of the system with the mobile phase for 9 min prior to subsequent analysis. In addition, by increasing the lipophilic nature of the mobile phase compared to that reported by Wright *et al.* [3], the run time was reduced by approximately 50% from 30 to 15 min while maintaining virtual baseline separation of the analytes. Overall, therefore, a series of 50 samples could be analyzed in approximately 16.75 h. Finally, it was noted that the need for a second extraction step as previously reported [3,10] was not necessary when EOA was used to reduce the amount of unreacted ECF present in the mixture. When EOA was not added to the reaction mixture, the injection solvent was not completely miscible with the mobile phase. Failure to add EOA yielded chromatograms with unacceptable baseline noise and also limited the injection volume that could be introduced into the HPLC system.

Interestingly, although not previously reported, it was noticed that two steps during sample preparation required careful control. Firstly, the extraction from plasma was optimized using a 1 *M* solution of sulphuric acid rather than a 0.6 *M* solution as previously reported [3,10]. It was noted that by using 0.6 *M* sulphuric acid, the extraction of IB and I.S. was not consistent. However, by using 1 *M* sulphuric acid, samples consistently extracted to completion. Secondly, the evaporation step had to be carefully controlled, as it was noticed that the presence of even negligible heat resulted in the loss of drug from the sample tubes. This was, perhaps, expected as IB has a lower melting point (73°C) and is therefore more volatile compared to other NSAIDs including fenoprofen (168°C), ketoprofen (94°C) and flurbiprofen (110°C). This problem was solved by evaporating samples without heat. The evaporation step for 20 samples was complete in less than 25 min.

In conclusion, the above method is sensitive and convenient, allowing for large numbers of samples to be processed and run in a relatively

short period of time. Furthermore, the assay sensitivity allows for the measurement of IB enantiomers in plasma samples for extended periods of time following dosage administration, or when plasma sample size is limited.

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