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Short Communication

Improved high-performance liquid chromatographic assay method for the enantiomers of ibuprofen

Matthew R. Wright, Saeed Sattari, Dion R. Brocks and Fakhreddin Jamali

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta (Canada)

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ABSTRACT

A rapid, inexpensive and sensitive high-performance liquid chromatographic method for the quantitation of ibuprofen enantiomers from a variety of biological fluids is reported. This method uses a commercially available internal standard and has significantly less interference from endogenous co-extracted solutes than do previously reported methods. The method involves the acid extraction of drug and internal standard [(±)-fenoprofen] from the biological fluid with isooctane–isopropanol (95:5) followed by evaporation and derivatization with ethylchloroformate and *R*-(+)-α-phenylethylamine. Excellent linearity was observed between the peak-area ratio and enantiomer concentration ($r > 0.99$) over a concentration range of 0.25–50 μg/ml. This method is suitable for the quantitation of ibuprofen from single-dose pharmacokinetic studies involving either rats or humans.

INTRODUCTION

Ibuprofen (IB) is a 2-arylpropionate non-steroidal anti-inflammatory drug marketed as a racemate. Numerous studies have documented the pharmacokinetics of IB and described the marked unidirectional inversion of the *R*-enantiomer to the *S*-enantiomer [1]. There are a number of gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods available for the separation and quantitation of IB enantiomers from biological fluid samples (*e.g.* refs. 2–11). Many of these methods require lengthy precolumn derivatization procedures and sample preparation, or lack sufficient sensitivity. Recently a reversed-phase method using precolumn derivatization with *S*-(−)-1-(1-naphthyl)ethylamine was reported for the quantitation of IB enantiomers [12]. This method, however, resulted in the co-extraction of numerous endogenous compounds, some of which interfered with quantitation of IB enantiomers when columns with less than an optimal number of theoretical plates were used, and requires the use of an internal standard that is unavailable commercially. We report the development of an improved method for the quantitation of IB enantiomers from a variety of biological fluids using precolumn derivatization with *R*-(+)-α-phenylethylamine [*R*-(+)-α-PEA].

Correspondence to: Dr. F. Jamali, Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2N8, Canada.

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EXPERIMENTAL

Chemicals

Racemic IB and fenoprofen (internal standard) were obtained as gifts from Upjohn Canada (Don Mills, Canada) and Eli Lilly (Toronto, Canada), respectively. Optically pure *R*- and *S*-ibuprofen were generously provided by KaliChemie (Hannover, Germany). Ethyl chloroformate, isooctane (Omnisolv), acetonitrile (Assurance grade), hexane (Omnisolv) and isopropanol (Assurance grade) were obtained from BDH Chemicals Canada (Edmonton, Canada). *R*-(+)-α-PEA was obtained from Sigma (St. Louis, MO, USA). Triethylamine (TEA) and chloroform, both analytical grade, were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

Apparatus and chromatographic conditions

The HPLC apparatus (Waters, Mississauga, Canada) consisted of a Model 590 pump, a Model 481 variable-wavelength ultraviolet detector (set at 225 nm), a 710B Wisp autosampler, a 10 cm × 4.6 mm I.D. C_{18} analytical column packed with 5-μm particles (Phenomenex, Torrance, CA, USA), and a 2 cm × 4.6 mm I.D. Uptight precolumn (Upchurch Scientific, Rexdale, Canada) packed with 37–53 μm diameter reversed-phase material. Both the analytical column and precolumn were operated at ambient temperature. The recorder–integrator was a Model 3390A (Hewlett-Packard, Palo Alto, CA, USA).

Unless otherwise stated the mobile phase composition was acetonitrile–water–acetic acid–TEA (46.5:53.5:0.1:0.03, v/v), pH 4.9, and was pumped at a flow-rate of 1.6 ml/min at ambient temperature.

Normal-phase separation was also attempted using a 250 mm x 4.6 mm I.D. (Partisil 5-μm silica; Whatman, Rose Scientific, Edmonton, Canada). The mobile phase consisted of 0.5% isopropanol in hexane, and was pumped at 2 ml/min.

Standard solutions

Ethyl chloroformate (6 m*M*) was prepared in acetonitrile. The derivatizing reagent, *R*-(+)-α-PEA (0.5 *M*), was prepared in TEA–acetonitrile (2:8). Stock solutions of (±)-IB (100 μg/ml) and (±)-fenoprofen (25 μg/ml) were prepared in 10 ml of methanol and sufficient 0.01 *M* NaOH to make 100 ml of each solution. All solutions were stored at 5°C.

Sample preparation

To an aliquot of biological fluid (100 μl of rat plasma, 500 μl of human plasma, 100 μl of rat or human urine) were added 50 μl of internal standard solution [(±)-fenoprofen, 25 μg/ml], 200 μl of H_2SO_4 (0.6 *M*) and 3 ml of isooctane–isopropanol (95:5, v/v). The constituents were then vortex-mixed for 30 s followed by centrifugation at 1800 *g* for 5 min. The organic layer was transferred to clean tubes and evaporated to dryness (Savant Speed Vac concentrator–evaporator, Emerston Instruments, Scarborough, Canada). The residue was reconstituted in 200 μl of TEA in acetonitrile (50 m*M*), followed by the addition of 50 μl of ethyl chloroformate solution and vortex-mixing for 30 s. A 50-μl aliquot of *R*-(+)-α-PEA solution (0.5 *M*) was added, vortex-mixed briefly, and allowed to stand. After 2 min, 1 ml of HCl (0.25 *M*) was added followed by 3 ml of chloroform. The mixture was vortex-mixed for 30 s followed by centrifugation at 1800 *g* for 5 min. The aqueous layer was aspirated to waste and the remaining organic layer evaporated to dryness. The residue was reconstituted in 200 μl of mobile phase and aliquots of 10–150 μl were injected into the HPLC system.

Extraction efficiency

To assess the efficiency of the extraction of the enantiomers in the initial step, 0.5 ml of blank human plasma ($n = 3$) was spiked with 0.5 and 10 μg/ml IB. Following extraction with isooctane–isopropanol and evaporation the residue was reconstitued in 200 μl of mobile phase, and the peak area of underivatized IB was compared with that obtained after direct injection of IB.

To assess the extraction efficiency of the derivatized diastereomers a solution of IB in acetonitrile (100 μg/ml) was prepared, and aliquots were taken to provide solutions of 0.5 μg/ml ($n = 6$) and 10 μg/ml ($n = 6$) in a total volume of 200

μl. To each aliquot was added 50 μl of ethyl chloroformate solution, followed by vortex-mixing for 30 s, and 50 μl of *R*-(+)-α-PEA solution. The derivatization reaction in half of the tubes was terminated by the addition of 100 μl of water. To the other half of the tubes were added 1 ml of HCl (0.25 *M*) and 3 ml of chloroform, followed by vortex-mixing for 30 s and centrifugation at 1800 *g* for 5 min. The aqueous layer was aspirated to waste and the organic layer evaporated to dryness. The residue was reconstituted in 400 μl of mobile phase.

Aliquots of 10–40 μl were injected into the HPLC apparatus, and the peak areas corresponding to the *R*-(+)-α-PEA derivatives of *R*- and *S*-IB from the extracted ($n = 3$) and unextracted ($n = 3$) sets were compared to one another.

Derivatization yield

Plasma samples containing 10 μg/ml IB ($n = 10$) were extracted as outlined previously. The residue of half of the samples was reconstituted in 200 μl of mobile phase, and 10 μl were injected into the HPLC system. The remaining samples were derivatized, extracted and reconstituted as outlined previously, and 10 μl were injected into the HPLC apparatus. The peak areas corresponding to underivatized IB observed in both sets of samples were compared to estimate the derivatization yield.

Accuracy and precision

Aliquots of biological fluid were spiked with racemic IB to yield concentrations of 0.25, 0.5, 1, 2, 5 and 10 μg/ml of each enantiomer. The solutions were analyzed according to the procedure described previously. Each solution was prepared in triplicate on three consecutive days. Accuracy was calculated as the mean % error, [(mean measured concentration − expected concentration)/expected concentration x 100%], while precision was evaluated by calculation of the inter- and intra-day coefficients of variation (C.V.).

Pharmacokinetic studies in rats and humans

Four male Sprague–Dawley rats (weight 284 ± 47 g) with a jugular vein cannula in place received an intravenous 5 mg/kg dose of racemic IB in PEG 400. Blood samples (0.2 ml) were collected from the jugular vein cannula at 0, 0.167, 0.333, 0.5, 1, 2, 3, 4, 6, 8 and 24 h. After each sample the cannula was flushed with heparinized saline (100 U/ml). Each blood sample was immediately centrifuged and the plasma separated and frozen until analysis.

A healthy male subject (24 years) received a single 400-mg dose of racemic IB (Advil, Whitehall-Robins, Mississauga, Canada) following dental extraction. Blood samples were taken predose and 15, 30, 45, 60, 120, 270 and 330 min following the dose. The blood was centrifuged and the plasma removed and kept frozen until analysis.

RESULTS AND DISCUSSION

Representative chromatograms of blank samples, drug-spiked samples and *in vivo* samples are shown in Fig. 1. The elution order of the IB enantiomers was confirmed by extraction and chromatography of optically pure *R*- and *S*-IB. Using these samples it was shown that the derivative of *R*-IB eluted before the derivative of *S*-IB. Therefore, the peaks eluting at 11.70 and 13.40 min correspond to the internal standard diastereomers, while those at 15.69 and 17.65 min correspond to the diastereomers of *R*- and *S*-IB, respectively. Both of the peaks derived from IB and the first internal standard peak (retention time 11.70 min) were free of interference from endogenous substances.

The efficiencies of extraction of both the enantiomers of IB and of the diastereomers formed upon derivatization were nearly complete (103 ± 8 and 101 ± 10%, respectively). Furthermore, derivatization was also essentially complete (94.3 ± 7.3%) in under 4 min.

Excellent linearity ($r > 0.99$) was observed between the peak-area ratios of the diastereomers of *R*- and *S*-IB to the first internal standard peak and the corresponding enantiomer concentration. A typical plasma standard curve could be described by $y = 0.6205x + 0.23$ ($r = 0.998$, $n =$

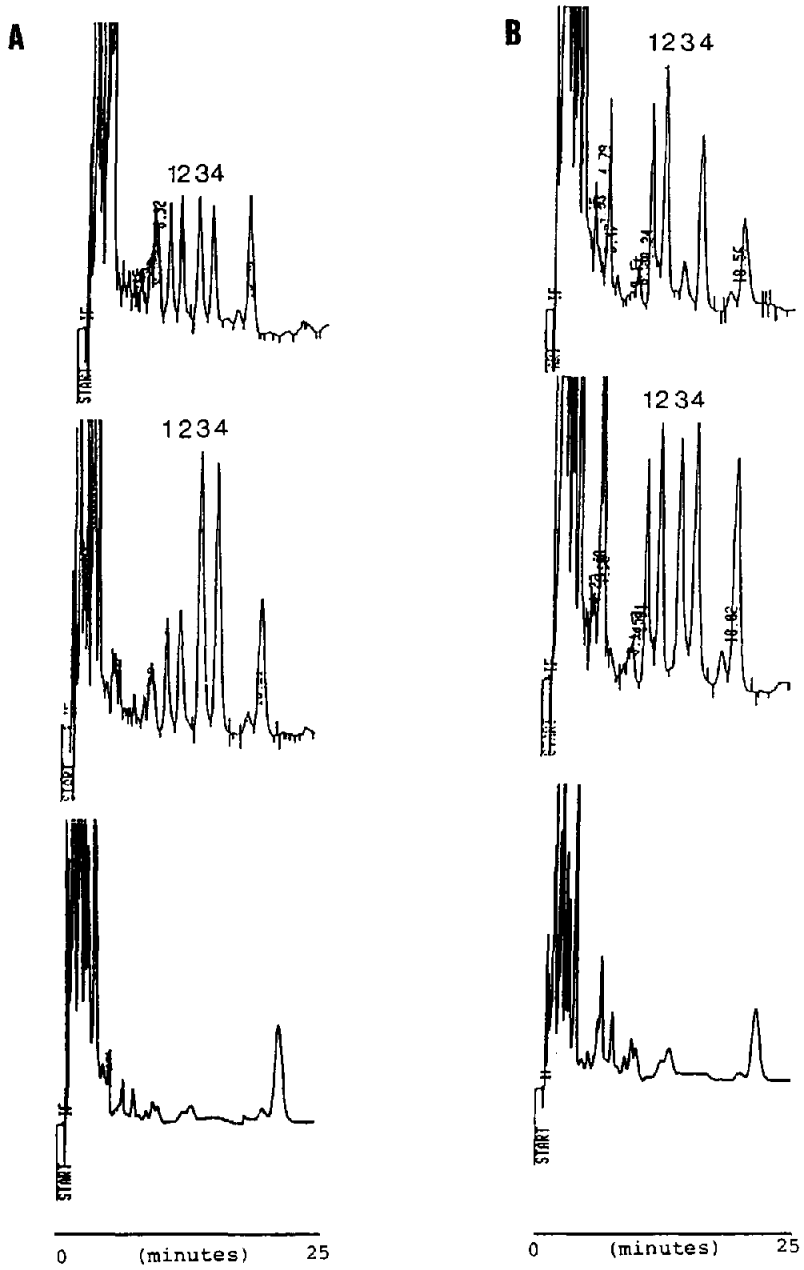

Fig. 1. Representative chromatograms from (A) human plasma and (B) rat plasma. In each case the respective chromatograms are (bottom) blank plasma, (middle) drug-spiked plasma and (top) plasma from a dosed subject. Peaks: 1 = *R*-fenoprofen; 2 = *S*-fenoprofen; 3 = *R*-IB; 4 = *S*-IB. The concentrations of IB enantiomers are 1.0 μg/ml (*R*-IB) and 0.88 μg/ml (*S*-IB) and 5 μg/ml of each enantiomer in the human dosed and spiked samples, respectively. The concentrations of IB enantiomers are 1.1 μg/ml (*R*-IB) and 4.9 μg/ml (*S*-IB) and 10 μg/ml of each enantiomer in the rat dosed and spiked samples, respectively.

9) and $y = 0.6826x + 0.27$ ($r = 0.998$, $n = 9$) for *R*- and *S*-IB, respectively (where y is the peak-area ratio and x is the enantiomer concentration) over a concentration range of 0.25–50 μg/ml of each enantiomer. As demonstrated in Table I, the method is accurate and precise, over a concentra-

TABLE I

ACCURACY AND PRECISION

n = 9 for each concentration.

Concentration (μg/ml)			Absolute error (%)		Inter-day C.V. (%)	
Expected	Measured					
	R-IB	*S*-IB	*R*-IB	*S*-IB	*R*-IB	*S*-IB
0.25	0.239	0.212	5.0	15.0	4.0	13.0
0.5	0.499	0.455	0.3	9.0	10.0	10.0
1	1.029	1.004	−2.9	−0.4	3.0	1.0
2	2.056	2.030	−2.8	−1.5	5.0	2.0
5	5.098	5.220	−2.0	−4.4	5.0	3.0
10	9.937	9.886	0.6	0.1	6.0	6.0

tion range of 0.25–10 μg/ml. The mean absolute error ranged from 0.1 to 15% with inter-day C.V. ranging from 1 to 13%.

Figs. 2 and 3 show the plasma concentration *versus* time profiles for a male human subject and four male Sprague–Dawley rats receiving oral and intravenous doses of racemic IB, respectively. Calculation of meaningful estimates of the pharmacokinetic parameters describing the plasma concentration *versus* time profiles is difficult due to the significant proportion of *R*-IB unidirectionally converted to *S*-IB [13]. However, there is marked stereoselectivity in the plasma concentrations of *R*- and *S*-IB in both species.

In addition, the concentrations of *R*- and *S*-IB determined by the present method were compared to those determined by the method of Mehvar *et al.* [12]. Fig. 4 shows the correlation of the measurements made by each method. The relationship between the concentrations determined by the two methods can be expressed as $y = 1.02x + 0.01$ ($r = 0.972$, $n = 14$) and $y = 0.93x + 0.064$ ($r = 0.972$, $n = 14$) for *R*- and *S*-IB, respectively, where y is the concentration of

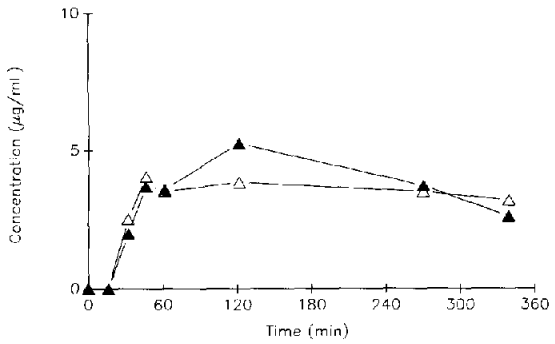

Fig. 2. Plasma concentration *versus* time profiles of *R*-IB (△) and *S*-IB (▲) in a dental patient following administration of 400 mg of racemic IB.

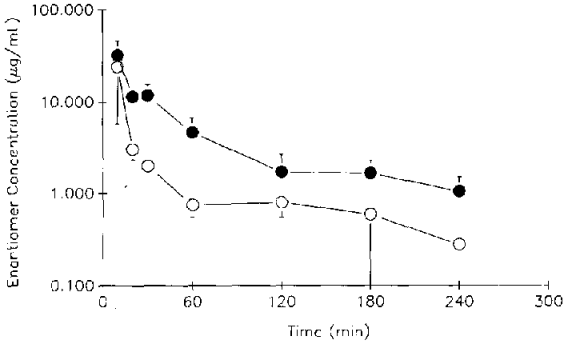

Fig. 3. Mean (± S.D.) plasma concentration *versus* time profiles of *R*-IB (○) and *S*-IB (●) in four male Sprague–Dawley rats following intravenous administration of 5 mg/kg racemic ibuprofen.

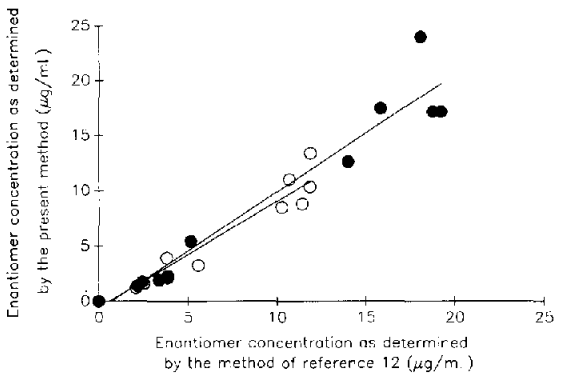

Fig. 4. Correlation between *R*-IB (●) and *S*-IB (○) plasma concentrations as determined by the present method and that of ref. 12.

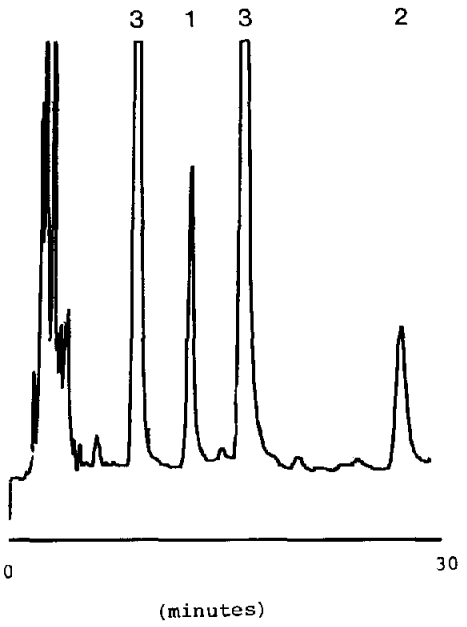

Fig. 5. Representative chromatogram of the normal-phase separation of *R*- and *S*-IB in human plasma. Peaks: 1 = *S*-IB; 2 = *R*-IB; 3 = (±)-fenvalerate (internal standard). The unidentified peak at 60 min is not shown.

IB enantiomer determined by the present method and x is the concentration as determined by the method of Mehvar *et al.* [12]. The mean percentage differences between the concentrations measured by the two methods were 1.7 and −2.3% for *R*- and *S*-IB, respectively.

The present method offers significant advantages over those previously reported since many of these require long precolumn derivatization times [2–4,8], result in the co-extraction of significant amounts of endogenous material [12], require the use of a commercially unavailable internal standard [12] or involve the use of relatively expensive chiral HPLC columns [7,9,11]. Further the present method employs relatively simple HPLC technology, *viz.* reversed-phase column and isocratic elution. As well as providing derivatives which are resolvable under reversed-phase conditions, *R*-(+)-α-PEA derivatives of ibuprofen are also resolvable under normal-phase conditions (resolution factor > 10) (Fig. 5). The diastereomers eluted at 12 and 26 min and were free of interfering components from human plasma. There was a drawback to this procedure, however, because of late-eluting peaks which appeared in the chromatograms up to 60 min after the injection into the HPLC system. This problem might have been resolvable using gradient elution.

Using the present method large numbers of biological samples may be analysed in a relatively short period of time. For example, fifty samples from biological fluids may be prepared for analysis in approximately 2.5 h with an HPLC runtime of 21 h. Thus, the developed method is rapid, relatively inexpensive and sensitive enough for use in single-dose pharmacokinetic studies in both rats and humans.

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