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Report

Stereospecific High-Performance Liquid Chromatographic (HPLC) Assay of Fenoprofen Enantiomers in Plasma and Urine[1]

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A new high-performance liquid chromatographic (HPLC) assay suitable for pharmacokinetic studies of enantiomers of fenoprofen (FEN) was developed. Following the addition of internal standard (IS; racemic ketoprofen), the plasma or urine constituents are extracted into a mixture of isooctane:isopropanol (95:5), back extracted into water, and finally, extracted into chloroform. After evaporation of the organic layer, the drug and IS are derivatized with *l*-leucinamide hydrochloride via ethyl chloroformate intermediate. The formed diastereoisomers are chromatographed on a reversed-phase HPLC with a mobile phase consisting of monopotassium phosphate solution:acetonitrile:triethylamine (65:35:0.02) at a flow rate of 1 ml/min. The detection UV wavelengths are 232 and 275 for the drug and IS, respectively. The suitability of the assay for pharmacokinetic analysis of FEN enantiomers was examined by analysis of the plasma and urine samples taken from a healthy subject, following peroral administration of a single 300-mg dose of racemic FEN.

KEY WORDS: fenoprofen; high-performance liquid chromatography (HPLC); nonsteroidal antiinflammatory drugs (NSAID); fenoprofen enantiomers; diastereoisomer separation; stereospecific analysis.

INTRODUCTION

Except for naproxen, the substituted, 2-arylpropionic acid nonsteroidal antiinflammatory drugs (NSAIDs) are marketed and used as racemic mixtures of S and R enantiomers. While *in vitro* data showed greater activity for the S isomer, the *in vivo* activities of the enantiomers of some NSAIDs were very close to one another (1). This has been attributed to a bioinversion of the relatively inactive R isomer to the S antipode (1).

Fenoprofen (FEN) is a chiral NSAID which undergoes a very extensive R-to-S inversion in humans (2). Furthermore, both enantiomers undergo ring hydroxylation (2). Reported stereospecific chromatographic methods capable of analyzing the drug enantiomers are scarce (2–4) and consist of lengthy sample preparation. Precolumn derivatization of the carboxylic moiety of indoprofen (5) and ketoprofen (6) with *l*-leucinamide and subsequent resolution of the resultant distereoisomers utilizing reversed-phase high-performance liquid chromatography (HPLC) have already been reported. In this article, application of the same derivatization technique to analysis of the FEN enantiomers is reported.

MATERIALS AND METHODS

Chemicals

Racemic powders of fenoprefen calcium and internal standard (IS), racemic ketoprofen, were gifts from Eli Lilly (Toronto, Canada) and Rhone-Poulenc (Montreal, Canada), respectively. Ethyl chloroformate was obtained from BDH Chemicals (Edmonton, Canada), and *l*-leucinamide hydrochloride was purchased from Sigma Chemical Company (St. Louis, Mo.). Acetonitrile and water were HPLC grade, while triethylamine (TEA), acetic acid, isopropanol, isooctane, chloroform, and sulfuric acid were analytical grade.

Apparatus and Chromatographic Conditions

The HPLC (Waters Scientific, Mississauga, Ontario) consisted of a 590 pump, a 490 programmable multiwavelength UV detector, a 710B Wisp autosampler, a 4.6-mm × 10-cm analytical column containing 5-μm octadecylsilane packing material (Partisil 5 ODS-3; Whatman, Clifton, N.J.), and a 2-cm Uptight Precolumn (Upchurch Scientific, Rexdale, Ontario) packed with 37 to 53-μm reversed-phase material. The recorder was a Hewlett Packard Model 3390A integrator (Mississauga, Ontario).

The detector was set at 275 nm for the first 13 min and then switched to 232 nm.

The mobile phase, unless stated otherwise, consisted of monobasic potassium phosphate (0.07 *M*):acetonitrile:TEA (35:65:0.02) with a pH of 6.0 which was pumped at flow

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rates of 1 and 1.2 ml/min, for plasma and urine samples, respectively.

Standard Solutions

Ethyl chloroformate (60 m*M*) and *l*-leucinamide (1*M*) solutions were prepared in acetonitrile, and 1 *M* TEA in methanol, respectively. Stock solutions of FEN calcium were prepared in distilled water, while the IS was dissolved in a 0.01 *M* solution of NaOH. Blank plasma and urine were spiked with standard solutions of the drug to contain final enantiomeric FEN concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 20 mg/liter for plasma and 1.0, 2.5, and 20 mg/liter for urine.

Sample Preparation

To 0.5 ml of plasma containing FEN were added 0.05 ml of IS (0.05 mg/ml racemic KT) and 0.1 ml of sulfuric acid (0.6 *M*). The constituents were then extracted with 3 ml of a mixture of isooctane:isopropanol (95:5) following vortex mixing for 30 sec and centrifuging (Adams Dynac Centrifuge; Clay-Adams, Parsipanny, N.J.) at 3000 rpm for 5 min. The organic layer was transfered to clean tubes and 2.5 ml of HPLC water was added. Following vortex mixing for 15 sec and centrifuging for 3 min, the organic layer was discarded. The drug and IS were then back extracted to 2.5 ml chloroform after acidification of the aqueous layer with 0.2 ml 0.6 *M* sulfuric acid and vortex mixing and centrifuging of the resultant for 15 sec and 3 min, respectively. The aqueous layer was discarded and the organic layer was evaporated to dryness (Savant Speed Vac Concentrator/Evaporator; Emerston Instruments, Scarborough, Ontario).

The FEN conjugates in urine samples (0.5 ml) were hydrolyzed instantaneously by the addition of 0.25 ml 1 *M* NaOH at room temperature; this method has previously been utilized for hydrolysis of ester conjugates of NSAIDs such as ketoprofen (6), etodolac (7), tiaprofenic acid (8,9), and naproxen (10). The samples were then acidified by the addition of 0.3 ml 0.6 *M* sulfuric acid and extracted in the same way as described for the plasma samples.

Following reconstitution of the residue in 0.1 ml of 50 m*M* TEA in acetonitrile, 0.05 ml of ethyl chloroformate and 0.05 ml of *l*-leucinamide solutions were added to the solution, at 30-sec intervals. Two minutes after the addition of *l*-leucinamide, 0.05 ml of distilled water was added to this mixture and aliquots of 0.01 to 0.04 ml of the solution were injected into the HPLC.

Extraction Yield

Plasma samples ($N = 5$) spiked with 1 and 10 mg/liter of R- and S-FEN were extracted according to the procedure mentioned above, but without the addition of IS. Exact volumes of the organic and aqueous layers were removed, and the peak areas of the racemic FEN after injection of the extracted samples were compared with those obtained after injection of the unextracted samples containing equivalent concentrations of the drug. The analysis of the racemic FEN was carried out under the chromatographic conditions described above but with a different mobile phase, which consisted of 0.07 *M* monopotassium phosphate:acetonitrile:TEA (70:30:0.02).

Derivatization Yield

Plasma samples ($N = 10$) spiked with 20 mg/liter of each FEN enantiomers were extracted in accordance with the mentioned procedure. Half of the samples were then derivatized to the amide derivatives, while the other half were only dissolved into the same volume of the solvent used in the preparation of the reagents. The derivatization yield was calculated by comparing the peak areas of the racemic FEN in the derivatized and underivatized samples. The analysis was carried out using the method applied for determination of the extraction yield.

Accuracy and Precision

Various concentrations of FEN (Table I) were added to plasma ($N = 6$) and the individual isomers were quantified against standard curve. The accuracy of the method was calculated based on the difference between the mean calculated and the mean added concentrations, while the precision was evaluated by calculating interday CVs.

RESULTS AND DISCUSSION

Precolumn derivatization of the carboxylic acid moiety with chiral reagents and subsequent chromatographic resolution of the resultant diastereoisomers have been reported for chiral NSAIDs (2–6). The few reported methods capable of analyzing FEN enantiomers utilize phenylethylamine (4) or amphetamine (2,3) as the derivatizing reagent. The derivatization reactions, however, are lengthy and, in some instances (3,4), take place only after exposure to high temperatures. The application of the derivatization method used for analysis of indoprofen (5) and ketoprofen (6) to FEN, how-

Table I. Accuracy and Precision of the Method ($N = 6$)

Theoretical conc. (mg/liter)	Measured conc. (mg/liter), mean (range)		% error		Interday % CV	
	R-FEN	S-FEN	R-FEN	S-FEN	R-FEN	S-FEN
0.25	0.285 (0.247–0.323)	0.281 (0.250–0.316)	14.0	12.6	6.9	8.9
0.50	0.525 (0.485–0.586)	0.519 (0.496–0.539)	5.1	3.8	7.4	3.6
1.0	1.08 (1.05–1.14)	1.12 (1.07–1.16)	8.5	11.7	2.9	3.8
2.5	2.51 (2.42–2.62)	2.50 (2.40–2.56)	0.28	−0.1	1.3	2.3
5.0	4.95 (4.82–5.12)	4.90 (4.82–5.07)	−1.0	−1.9	1.8	2.3
10	9.77 (9.69–9.94)	9.69 (9.50–9.78)	−2.3	−3.1	1.4	1.1
20	20.1 (19.4–20.8)	20.2 (19.4–20.5)	0.64	0.80	2.7	2.2

ever, resulted in a very efficient and rapid derivatization. The reaction takes place at the ambient temperature in a very short period of time (less than 3 min) with 95.7 ± 3.7% overall efficiency.

The overall extraction efficiencies of FEN from plasma samples were 84 ± 4.4 and 92 ± 4.4% for 1 and 10 mg/liter enantiomeric FEN concentrations, respectively. Under the conditions stated for determination of extraction and derivatization yield, the racemic FEN eluted as a sharp peak with a retention time of 10.7 min.

Extraction of both plasma and urine and subsequent derivatization of the samples with *l*-leucinamide resulted in chromatograms free of interfering peaks (Figs. 1 and 2). In plasma, the derivatives of R- and S-FEN were separated with a resolution factor of 1.99 and eluted with retention times of 19.1 and 22.0 min, respectively. Due to unavailability of the pure isomers of FEN, the configurations were assigned based on the relative magnitudes of the enantiomers in the plasma of a healthy male volunteer who took a single 300-mg po dose of FEN (Nalfon; Eli Lilly, Canada). The plasma concentration of the S isomer has been reported to be substantially greater than that of the R isomer, due to the R-to-S bioinversion (2). The order of elution of ketoprofen isomers, on the other hand, was determined by

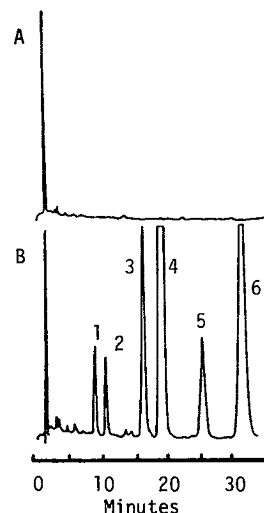

Fig. 2. Chromatograms of a blank urine sample (A) and a 0- to 2-hr urine sample of a subject after a single 300-mg oral dose of racemic fenoprofen (B). Peaks: 1 and 2, internal standard diastereoisomers; 3 and 4, diastereoisomers of R- and S-fenoprofen, respectively; 5 and 6, unknown metabolites.

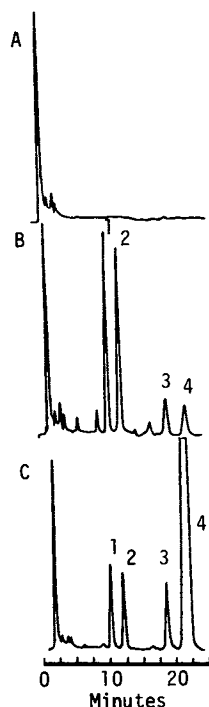

Fig. 1. Chromatograms of blank plasma (A), blank plasma spiked with 0.25 mg/liter of each enantiomer (B), and a plasma sample taken from a healthy subject 3 hr after oral administration of a single 300-mg dose of racemic fenoprofen (C). Peaks: 1 and 2, internal standard diastereoisomers; 3 and 4, diastereoisomers of R- and S-fenoprofen, respectively.

derivatization and injection of optically pure S isomer (6). The diastereoisomers of ketoprofen also eluted with an order similar to that of FEN and were resolved with a factor of 1.84. Retention times of the derivatives of R- and S-FEN in urine were 16.3 and 19.1 min, respectively.

In urine, after alkaline hydrolysis, in addition to the derivatized R- and S-FEN, there were two other peaks with retention times of 25.4 and 31.6 min (Fig. 2). Using a gas chromatographic (GC) method, Rubin *et al.* (2) were able to detect relatively large amounts of the conjugated S and small amounts of the conjugated R 4'-hydroxy metabolite of FEN in urine. The two unknown peaks, observed in the volunteer's urine chromatogram (Fig. 2), therefore might represent hydrolyzed conjugates of hydroxy metabolites of the FEN enantiomers. These investigators were not able to detect any conjugated R-FEN in the urine of the subjects after a 600-mg racemic dose of FEN. The greater sensitivity of our method, on the other hand, permits analysis of both enantiomers in plasma and urine after the administration of a single 300-mg racemic dose.

The within-the-run change of the wavelength was essential, as some endogenous peaks coeluted with IS, at 232 nm. Sallustio *et al.* (4) also noticed interfering peaks when analyzing FEN and ketoprofen in plasma. They, therefore, used a lengthy procedure which involved reversed-phase chromatography of the extracted plasma, collection of the drug, derivatization of the enantiomers, and then normal-phase chromatography of the derivatives.

Excellent linearity was observed between the peak area ratios (R- and S-FEN/IS) and the corresponding plasma and urine concentrations in the examined concentration range ($r > 0.999$). For the sake of consistency, in all instances derivatized R-ketoprofen was used for quantification. A typical

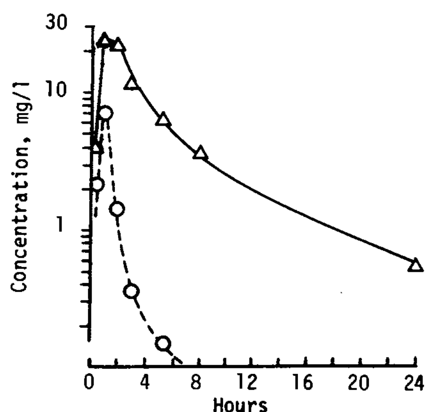

Fig. 3. Plasma concentration–time profiles of S (△)- and R (○)-fenoprofen in a healthy subject following oral administration of a single 300-mg dose of racemic fenoprofen.

plasma standard curve could be described by $y = 0.0055 + 1.5301x$ and $y = -0.0098 + 1.5052x$ for the R and S enantiomers, respectively. As reflected by the slopes of the best-fit lines for R and S isomers, the responses to both enantiomers were very close, indicating no stereoselectivity in the extraction or the derivatization methods. This, however, was not the case for the method reported by Sallustio *et al.* (4), as their method showed stereoselectivity for the R-FEN over the S enantiomer.

The assay is accurate and reproducible as reflected by the interday CV values and the differences between the added and the found concentrations (Table I). In terms of sensitivity, the lowest examined plasma concentration (0.25 mg/liter) was analyzed (Fig. 1) with an error of 14.0 and 12.6% and an interday variation of 6.9 and 8.9% for the R and S isomers, respectively. If the criterion of signal:noise ratio were used, a sensitivity of far better than 0.25 mg/liter could be claimed (Fig. 1). The few reported assays possess a lower sensitivity; the minimum quantifiable concentration in one of the methods was reported to be 2.5 mg/liter (4).

Plasma time courses of the isomers of FEN are depicted in Fig. 3. The decline in plasma concentrations of the more active isomer was substantially slower than that of the less active one. This can be attributed, at least partly, to an extensive R-to-S bioinversion (2).

In conclusion, the above method is very rapid, sensitive, and convenient and is suitable for pharmacokinetic analysis of the FEN enantiomers after administration of the recommended therapeutic doses.

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