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Short Communication

Enantiospecific analysis of ketoprofen in plasma by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic (HPLC) assay for the determination of the *R*- and *S*-enantiomers of ketoprofen is described. Facile ketoprofen extraction from plasma and derivatization to the diastereomeric *S*-1-phenylethylamides was followed by normal-phase HPLC. The ketoprofen diastereomeric amides eluted within 8 min. The limit of quantification of the assay was 0.15 mg/l of each enantiomer (signal-to-noise ratio = 5).

INTRODUCTION

Ketoprofen, 2-(3′-benzoylphenyl)propanoic acid, is a member of the 2-arylpropanoic acid series of non-steroidal anti-inflammatory drugs. Ketoprofen is marketed as the racemate; however, in keeping with other studied members of this structural drug class, it exhibits enantioselective pharmacodynamic and pharmacokinetic properties (recently reviewed by Jamali and Brocks [1]). The importance of discriminating between the separate enantiomers of this drug and of other 2-arylpropanoates, when studying the pharmacology in human subjects, has become self-evident [2].

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Earlier reports of analytical methods for ketoprofen in biological matrices utilized achiral techniques for the determination of unresolved drug [3–5]. Previous enantiospecific high-performance liquid chromatographic (HPLC) methods [6–8] entailed either extensive sample preparation or lengthy chromatography times. Sallustio *et al.* [6] utilized a protracted sample clean-up, employing reversed-phase HPLC isolation and quantification of unresolved ketoprofen followed by derivatization and subsequent normal-phase HPLC analysis of the diastereomeric *S*-1-phenylethylamides of ketoprofen.

Bjorkman [7] and Foster and Jamali [8] have developed reversed-phase HPLC methods for the L-leucinamide diastereomers of ketoprofen with single chromatographic run times of 16 and 20 min, respectively.

We report here major modifications to the method of Sallustio *et al.* [6] including simple extraction of unresolved drug from acidified plasma and subsequent normal-phase HPLC separation of the diastereomeric *S*-1-phenylethylamides within 8 min.

EXPERIMENTAL

Reagents and chemicals

Racemic ketoprofen was a gift from May and Baker (Sydney, Australia) and *S*-naproxen (internal standard) of F. H. Faulding (Adelaide, Australia). Pure *R*- and *S*-ketoprofen were kindly supplied by Dr. Kathy Knights of The Flinders University of South Australia (Bedford Park, Australia). Racemic [1-^{14}C]ketoprofen was synthesized and authenticated [9]. HPLC-grade heptane, isopropyl alcohol and methanol were purchased from BDH (Poole, UK) as were analytical-grade sulphuric acid, *n*-hexane, ethyl acetate and dichloromethane for sample extraction. Thionyl chloride (redistilled over linseed oil within four weeks of use) was purchased from May and Baker (Dagenham, UK), sodium hydroxide and linseed oil were from Ajax (Sydney, Australia) and *S*-1-phenylethylamine was obtained from Sigma (St. Louis, MO, USA). Racemic fenoprofen calcium was obtained from Eli Lilly (Indianapolis, IN, USA), racemic ibuprofen was a kind gift of Boots (Nottingham, UK), mefenamic acid was obtained from Parke Davis (Sydney, Australia) and salicylic acid from F. H. Faulding.

Instrumentation and chromatographic conditions

The liquid chromatograph consisted of a Model 510 pump, Wisp autoinjector, Model 490 variable-wavelength UV absorbance detector and Model 840 data station, all from Waters Assoc. (Milford, MA, USA).

An SGE (Sydney, Australia) glass-lined HPLC column (250 mm × 4 mm I.D.) containing 5 μm silica was operated at ambient temperature (22°C) through which the mobile phase (isopropyl alcohol–*n*-heptane, 8:92, v/v) was pumped at 1 ml/min. This solvent was filtered (0.22 μm) and degassed immediately prior to use and an in-line 2 μm filter (Waters Assoc.) was positioned ahead of the column.

The column was monitored for UV absorbance at a detection wavelength of 254 nm.

Sample preparation and derivatization

In a culture tube (100 mm × 16 mm) equipped with a PTFE-lined screw cap were added 1.0 ml of plasma, 0.05 ml of internal standard solution (*S*-naproxen, 200 mg/l in methanol), 0.5 ml of 2 *M* sulphuric acid and 8 ml of extracting solvent (10% ethyl acetate in *n*-hexane). Each sample was gently mixed for 10 min on a rotary mixer (30 rpm) and then centrifuged for 10 min (1500 *g*). The organic layer was transferred to a fresh culture tube and evaporated to dryness at 45°C under a stream of purified nitrogen (Zymark, Hopkinton, MA, USA). The dried residue was reconstituted with 0.1 ml of 1.5% thionyl chloride in *n*-hexane (freshly prepared) and the tube firmly capped and heated for 1 h at 75°C in a dry heat bath. The sample was subsequently allowed to cool to room temperature before adding 0.5 ml of 2% *S*-1-phenylethylamine in dichloromethane (freshly prepared) and re-capping the tube for a further 15 min. Final extraction of the lipophilic amides was accomplished by the addition of 0.5 ml of 2 *M* sulphuric acid and 5 ml of *n*-hexane. Mixing, centrifugation and drying of the organic layer were as described above for the initial extraction step. The dried residue was reconstituted with 0.25 ml of mobile phase and 0.2 ml was injected onto the HPLC column.

Calibration, precision and accuracy

Racemic ketoprofen plasma standards were prepared by adding 1.0 ml of drug-free plasma to culture tubes containing dried methanolic extracts of ketoprofen such that the final concentration range of 0.156–10.00 mg/l for each enantiomer was achieved. These standards were taken through the sample preparation and derivatization methods described above. Separate calibration curves for each ketoprofen enantiomer were constructed as the peak-area ratios of the diastereomers to the internal standard diastereomer, and least-squares linear regression analysis was performed to determine slopes, intercepts and regression coefficients.

The concentration/normalised peak-area ratios for each enantiomeric ketoprofen standard over the concentration range were also calculated for each set of standards.

The accuracy and precision of the method were assessed by preparing methanolic solutions of racemic ketoprofen from weighings independent of those used for preparing the calibration standards. Addition of drug-free plasma to dried methanolic extracts yielded ketoprofen enantiomeric concentrations in plasma of 0.200 and 9.00 mg/l. Aliquots (1 ml) of these plasma samples were analysed to determine intra-day accuracy and precision, and inter-day (eight-week period) accuracy and precision of the assay.

Extraction efficiency

In order to optimize and quantitate the initial extraction of unresolved ketoprofen enantiomers and internal standard from plasma a reversed-phase non-stereoselective HPLC method was used [9]. Briefly, this achiral method involved simple extraction of ketoprofen and internal standard from acidified plasma and chromatography on a radially compressed (RCM-100) phenyl cartridge (4 μm, 100 mm × 8 mm I.D., Waters Assoc.) through which the mobile phase (acetonitrile–10 m*M* acetate buffer, pH 3, 50:50, v/v) was pumped at 2 ml/min (UV wavelength detection at 260 nm).

Instrumentation was identical to that described above for the enantiospecific ketoprofen assay.

The peak areas after injection of unresolved ketoprofen and internal standard extracted from plasma were compared to those generated from direct injections of aqueous solutions of these compounds. Extraction efficiency was assessed at the upper and lower ends of the calibration range (18 and 0.4 mg/l unresolved racemic ketoprofen) and of the internal standard.

Derivatization efficiency

Racemic [1-^{14}C]ketoprofen was synthesized and subsequently purified by the non-stereoselective reversed-phase HPLC method detailed under *Extraction efficiency* (described in detail elsewhere [9]). A methanolic aliquot of this radiochemically pure ketoprofen (specific activity 8.70 mCi/mmol) was added to drug-free plasma to achieve an enantiomeric concentration of 1.25 mg/l and taken through the enantiospecific assay extraction and derivatization steps. The HPLC eluates corresponding to the [1-^{14}C]-*R*- and [1-^{14}C]-*S*-ketoprofen diastereomeric amide peaks were collected in glass tubes, evaporated to dryness under nitrogen (45°C), reconstituted and counted in a xylene-based liquid scintillant (PCS II, Amersham, U.K.).

Counting was carried out in a Nuclear-Chicago (Des Plaines, IL, USA) liquid scintillation system (Unilux III) and quench correction was performed with the internal standard method ([^{14}C]toluene, Amersham). These counts were compared with counts from pure radiolabelled racemic ketoprofen. The combined extraction and derivatization efficiency for ketoprofen could thus be determined together with a measure of potential chiral discrimination during the derivatization reaction between enantiomeric ketoprofen acyl chlorides and *S*-1-phenylethylamine.

Assigning absolute configuration

Each authentic pure enantiomer of ketoprofen was taken through the complete sample preparation method to establish its retention time. Potential racemization was also checked by this method.

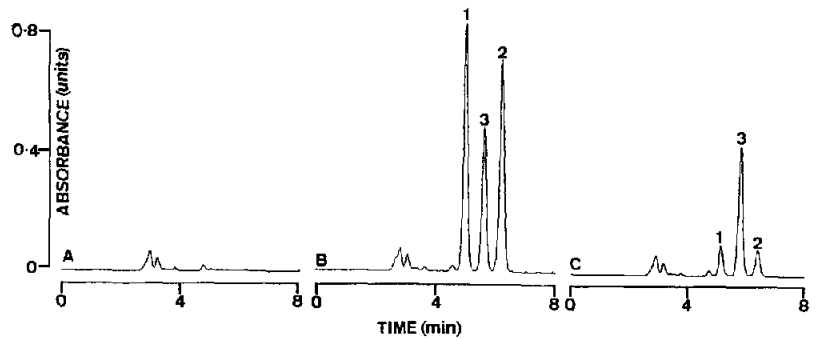

Fig. 1. Chromatograms of derivatized samples. (A) Extract of drug-free plasma (1 ml) without internal standard. (B) Plasma sample obtained 2 h after a 100-mg dose of racemic ketoprofen (Orudis, May and Baker) containing 6.21 mg/l *R*-ketoprofen and 4.82 mg/l *S*-ketoprofen. (C) Plasma sample obtained 6.5 h after this ketoprofen dose containing 0.521 mg/l *R*-ketoprofen and 0.484 mg/l *S*-ketoprofen. Peaks 1 and 2 represent the diastereomeric *S*-1-phenylethylamides of *R*-ketoprofen and *S*-ketoprofen, respectively, and peak 3 the *S*-1-phenylethylamide of *S*-naproxen (internal standard).

RESULTS AND DISCUSSION

Chromatograms resulting from the analysis of drug-free plasma and plasma obtained 2 and 6.5 h after a single 100-mg dose of racemic ketoprofen (Orudis, May and Baker, Sydney, Australia) are depicted in Fig. 1. No interfering endogenous plasma peaks were observed. Under the chromatographic conditions described for the enantiospecific assay, the retention times for the *R*- and *S*-ketoprofen diastereomeric amides were 5.2 and 6.6 min, respectively. *S*-Naproxen-*S*-1-phenylethylamide conveniently eluted between the ketoprofen peaks at 5.9 min.

Calibration curves generated over the enantiomeric concentration range from 0.156 to 10.00 mg/l (supplied as racemic drug) were linear for both enantiomers of ketoprofen. Linear least-squares regression analysis for twelve calibration

TABLE I

LINEAR LEAST-SQUARES REGRESSION ANALYSIS FOR TWELVE CALIBRATION CURVES FOR EACH KETOPROFEN ENANTIOMER CONSTRUCTED INDEPENDENTLY OVER AN EIGHT-WEEK PERIOD

Parameter	Value (mean ± S.D.)	
	R-Ketoprofen	*S*-Ketoprofen
Slope	2.87 ± 0.19	2.94 ± 0.21
y-Intercept	0.0120 ± 0.00054	0.0169 ± 0.00062
Regression coefficient	0.999 ± 0.0008	0.999 ± 0.0007
Concentration range (mg/l)	0.156 – 10.00	0.156 – 10.00

curves is depicted in Table I. In every case the correlation coefficient was >0.999.

The intra-day accuracy and precision of the assay was assessed by analysing six separate aliquots of two independently prepared plasma samples covering the upper and lower limits of the ketoprofen enantiomeric concentration range. For the first quality control sample containing 9.00 mg/l of each enantiomer, the mean concentrations of *R*- and *S*-ketoprofen were determined to be 9.25 mg/l (coefficient of variation, C.V. = 3.81%) and 9.08 mg/l (C.V. = 2.96%), respectively. The second sample contained 0.200 mg/l *R*-ketoprofen and *S*-ketoprofen. The concentrations determined were 0.217 mg/l (C.V. = 7.92%) and 0.193 mg/l (C.V. = 8.90%), respectively.

The inter-day accuracy and precision were determined by analysing the quality control plasma samples containing 9.00 and 0.200 mg/l of each enantiomer (supplied as the racemate) over an eight-week period. The mean (n = 12) concentrations of *R*- and *S*-ketoprofen for the lower plasma concentration samples were 0.191 mg/l (C.V. = 4.70%) and 0.196 mg/l (C.V. = 4.21%), respectively. The accuracy (and precision) values (n = 12) for the 9.00 mg/l plasma samples were 9.24 mg/l (C.V. = 3.90%) and 9.36 mg/l (C.V. = 5.00%) for the *R*- and *S*-enantiomers of ketoprofen, respectively.

The mean (±S.D.) extraction efficiency of 10% ethyl acetate in hexane–sulphuric acid (*vide supra*) was assessed (n = 6) for 18.0 mg/l racemic ketoprofen in plasma and found to be 83.9 ± 1.4% relative to an unextracted aqueous aliquot of ketoprofen utilizing the non-stereospecific assay described above. The efficiency of extraction (n = 6) for 0.400 mg/l racemic ketoprofen was 86.5 ± 3.3%. The respective value (n = 6) for the internal standard (*S*-naproxen) was 95.5 ± 2.9%.

Assessment of the combined extraction and derivatization methodologies of the enantiospecific assay entailed analysis of plasma sample containing 1.25 mg/l of each enantiomer of ketoprofen (supplied as purified racemic [1-^{14}C]ketoprofen) over a two-week period (n = 8). The mean (±S.D.) yield for unresolved ketoprofen was 79.6 ± 6.1% and the ratio of *R*- to *S*-ketoprofen diastereomeric amide counts was 1.002 ± 0.021. Thus, derivatization to the diastereomeric amides with *S*-1-phenylethylamine was non-stereoselective and largely quantitative.

Validation of the method in terms of potential racemization of ketoprofen enantiomers during sample processing was examined by analysis of plasma samples containing 5.00 mg/l pure *R*-ketoprofen and pure *S*-ketoprofen over a two-week period (n = 8 for each). The concentration of the contaminant antipode remained less than 2.5% in each case providing evidence that no racemization was taking place during sample extraction and derivatization.

It is important to establish the specificity of any analytical method which is designed for measurement of drug concentrations in biological matrices, since the possibility exists for subjects to be coadministered other drugs. Given the acidic environment in which extraction of xenobiotics takes place with this assay, a

range of acidic drugs were assessed in terms of their retention times relative to the elution profiles of ketoprofen and internal standard diastereomers. The retention times for the diasteromeric amides of the *R*- and *S*-enantiomers of ibuprofen were 3.3 and 4.1 min and for fenoprofen, 3.7 and 4.6 min, respectively. Mefenamic acid eluted as its respective amide at 3.0 min while salicylic acid had a retention time of 3.8 min.

In addition, plasma samples were obtained from a number of human subjects who had been prescribed a variety of xenobiotics not including non-steroidal anti-inflammatory agents. These included ranitidine, nifedipine, penicillamine, midazolam, diazepam, theophylline, methylprednisolone and digoxin. None of these parent compounds nor any observed metabolite peak coeluted with the *S*-1-phenylethylamides of ketoprofen and *S*-naproxen (internal standard).

In summary, we have described a rapid, sensitive and specific HPLC assay for the determination of ketoprofen enantiomers in plasma. The method performs with adequate accuracy and precision.

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