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Stereoselective Analysis of Ketorolac in Human Plasma by High-Performance Liquid Chromatography

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ABSTRACT A high-performance liquid chromatographic (HPLC) analytical method is described for the quantification of the (R)- and (S)-enantiomers of ketorolac when present together in human plasma. The method involves derivatization with thionyl chloride/(S)-1-phenylethylamine and subsequent reversed-phase chromatography of the diastereomeric (S)-1-phenylethylamides of (R)- and (S)-ketorolac. The method is suitable for the analysis of large numbers of plasma samples and has been applied in this report to a pharmacokinetic study of ketorolac enantiomers upon intramuscular administration of racemic drug to a human subject. The limit of quantification for each enantiomer of ketorolac is 50 ng/ml (signal-to-noise ratio > 10). © 1993 Wiley-Liss, Inc.

KEY WORDS: ketorolac enantiomers, HPLC, plasma concentrations, nonnarcotic analgesic, nonopiate analgesic, diastereomers, nonsteroidal antiinflammatory drugs (NSAIDs), stereoselective pharmacokinetics

Ketorolac (5-benzoyl-1,2-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid, see Fig 1) is a potent nonnarcotic analgesic whose major mechanism of action is via inhibition of prostaglandin synthesis.[1] Ketorolac is marketed for clinical use as the racemate and is prescribed for the short-term management of moderate to severe pain.[2,3] The (S)-enantiomer of ketorolac has been reported to be considerably more potent than its corresponding optical antipode in animal studies.[4] Thus the active enantiomer of ketorolac has the same absolute stereochemical configuration as other chiral α-substituted arylacetic acid antiinflammatory/analgesic agents.[5,6]

A number of achiral HPLC methods for the analysis of ketorolac in plasma have been documented in the literature.[7,8] These nonstereoselective analytical methods have been used to generate extensive human pharmacokinetic data for ketorolac without consideration for the stereochemistry of the drug.[7,9-11] The potential exists for misinterpretation of drug disposition data produced for racemic drugs using nonstereoselective assays.[12,13] To our knowledge only a single report (in abstract form) has defined the enantioselective disposition of this drug in man.[14] However, details of the analytical method used for determining enantiomer concentrations were not provided.

An earlier report describes the development and application of a HPLC method for the quantification of ketorolac enantiomers in rat plasma.[15] The method involved derivatization of ketorolac with ethyl chloroformate/(L)-leucinamide and subsequent reversed-phase chromatography of the diastereomers. The method was disadvantaged by long chromatography run times (up to 35 min per sample) and a lack of data pertaining to potential racemization of ketorolac during the derivatization procedure. Moreover, the method was applied to a pharmacokinetic study of rats dosed with approximately 10-fold the body weight corrected dose normally administered to humans.

We describe in this report the development and validation of a rapid, sensitive, and specific method for the quantification of ketorolac stereoisomers in human plasma upon administration of therapeutic doses of racemic drug. The method involves extraction of ketorolac and a structural congener [(S)-ketoprofen as internal standard] from acidified plasma, followed by derivatization with (S)-1-phenylethylamine via intermediate acid chloride generation. The diastereomers of (S)- and (R)-ketorolac are resolved with reversed-phase HPLC conditions within 12 min of injection. The limit of quantification of individual ketorolac enantiomers is 50 ng/ml. Application of the method enabled elucidation of the plasma concentration–time profiles for each stereoisomer upon intramuscular administration of a single 30 mg dose of (RS)-ketorolac to a human volunteer.

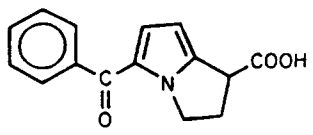

Fig. 1. Chemical structure of ketorolac.

MATERIALS AND METHODS

Chemicals

Racemic ketorolac free acid was extracted from ketorolac tromethamine solution for injection (Toradol®, Syntex, Sydney, Australia). To 25 ml of ketorolac tromethamine solution (30 mg/ml) was added 4 ml of 2 *M* sulphuric acid. This solution

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was extracted with ethyl acetate (150 ml) and the extract was washed with water, dried (anhydrous magnesium sulphate) and evaporated in vacuo. The solid was recrystallized from hexane-ethyl acetate. The (R)- and (S)-enantiomers of ketorolac were obtained by fractional recrystallization of the cinchonine and cinchonidine salts, respectively, of ketorolac from ethyl acetate.[4] The optical purity of (R)- and (S)-ketorolac obtained were each estimated to be approximately 98% as determined by the HPLC method described herein. (S)-Ketoprofen (optical purity of 99%) was a gift of Dr. Ralph Massy-Westropp (University of Adelaide, South Australia). (S)-1-Phenylethylamine (Lot: 10H3457, Sigma Chemical Company, St Louis, MO) was estimated to be approximately 99.5% optically pure as determined in a previous study[18] and used within 4 weeks of opening. Thionyl chloride (May and Baker, Dagenham, UK) was redistilled successively, over quinoline and boiled linseed oil prior to use. Spectroscopic-grade dichloromethane (Uvasol®, BDH Chemicals, Poole, UK) was stored over type 5A molecular sieves (BDH Chemicals). HPLC-grade acetonitrile, ethyl acetate, and *n*-hexane were purchased from BDH Chemicals. Triethylamine was obtained from Prolabo (Paris, France) and sodium acetate solution (20 mmol/10 ml) from David Bull Laboratories (Melbourne, Australia). HPLC-grade distilled water was furnished by a Modulab® laboratory water purification system (Water Systems Corporation, San Antonio, TX).

Sample Preparation and Derivatization

To a culture tube (100 × 16 mm) equipped with a teflon-lined screw cap were added 1.0 ml of plasma, 0.05 ml of internal standard solution [(S)-ketoprofen, 15 mg/liter in methanol:distilled water (1:4)], 0.075 ml of 2 *M* sulphuric acid, 0.10 ml of methanol:water (1:4), and 8.0 ml of 20% ethyl acetate in hexane. The tube was capped and the contents rotary mixed for 10 min after which the tube was centrifuged at 2,000*g* (10 min). The organic layer was transferred to a fresh culture tube and evaporated to dryness at 55°C under a stream of purified nitrogen (Zymark, Hopkinton, MA). The dried residue was reconstituted with 0.10 ml of 1.5% thionyl chloride in *n*-hexane (freshly prepared) and the tube was firmly capped, vortexed, and heated for 30 min at 80°C in a dry heat bath. Subsequently, the tube contents were cooled to room temperature upon which a 0.50 ml volume of 3.0% (S)-1-phenylethylamine in dry dichloromethane (prepared within 30 min of use) was added, the solution was vortexed, and left to stand at room temperature for 10 min. The contents of the tube were evaporated to dryness under a nitrogen stream, following which 1.0 ml of 2 *M* sulphuric acid and 5.0 ml of ethyl acetate were added. Mixing, centrifugation, transfer, and drying of the organic layer in a fresh glass culture tube were as described above for the initial extraction step. The dried residue was reconstituted with 0.125 ml of mobile phase and 0.10 ml was injected onto the HPLC column.

Chromatographic Instrumentation and Conditions

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

A radially compressed phenyl bonded-phase cartridge (Nova-Pak®, Waters Assoc., 4 μm, 100 × 8 mm i.d.) was eluted with 50% acetonitrile and 0.1% triethylamine in aqueous 20 m*M* sodium acetate buffer (final pH 5.5) at a flow rate of 2.0 ml/min (ambient temperature). The mobile phase was filtered (0.22 μm), sparged with helium immediately prior to use and pumped through an in-line 2 μm filter (Waters Assoc.) positioned ahead of the cartridge. The cartridge eluent was monitored for UV absorbance at two wavelengths (310 and 254 nm). Following injection of a derivatized sample, the wavelength was maintained at 310 nm for the first 8.0 min [sufficient time for elution of (S)- and (R)-ketorolac-(S)-1-phenylethylamides] and subsequently switched to 254 nm for detection of the later eluting (S)-ketoprofen-(S)-1-phenylethylamide (internal standard).

Calibration, Precision, and Accuracy

Racemic ketorolac plasma standards were prepared by adding 0.10 ml of methanol:distilled water (1:4) containing appropriate concentrations of (RS)-ketorolac to achieve a final concentration range of 50–3,200 ng/ml ($n = 7$) for each enantiomer (expressed in terms of the free acid). These standards were taken through the sample preparation and derivatization procedures described above with the exception that the addition of 0.10 ml of drug-free methanol:distilled water (1:4) was omitted. Separate calibration curves for each ketorolac enantiomer were constructed as the peak-area ratios of the derivatized enantiomers to the derivatized internal standard, and least-squares linear regression analysis was performed to determine slopes, intercepts, and regression coefficients. In addition, the concentration-normalized peak-area ratio was calculated for each ketorolac enantiomer standard within a given set of calibration standards.

The accuracy and precision of the method were assessed by analysing drug-free plasma spiked with solutions of (RS)-ketorolac in methanol:distilled water (1:4) prepared from weighings independent of those used for preparing the calibration standards. Aliquots (1 ml) of these plasma samples containing 75, 200, and 1000 ng/ml of each ketorolac enantiomer were assayed to determine intraday and interday accuracy and precision over a 4-week period.

Extraction and Derivatization Efficiency

A nonstereoselective HPLC method was used to optimize and quantitate the initial extraction of underivatized ketorolac from plasma. This method was based on identical chromatography conditions to those developed for unresolved ketoprofen,[16] with the exception that peak detection was carried out at a UV absorbance wavelength of 320 nm. The peak areas attributed to unresolved ketorolac following injection of both extracted and nonextracted samples were compared.

The extraction efficiency achieved following partitioning of the (S)-1-phenylethylamides of ketorolac between 2 *M* sulphuric acid (0.5 ml) and ethyl acetate (5 ml) was also assessed. The peak area of both (R)- and (S)-ketorolac diastereomeric amide were compared between extracted and nonextracted material.

The derivatization efficiency was determined by quantifying the proportion of underivatized ketorolac present in plasma samples spiked with racemic drug and taken through the sample preparation procedure. This analysis was carried out using the same nonenantioselective HPLC method applied for determination of the extraction yield of ketorolac from acidified plasma (vide supra).

Assigning Absolute Configuration

Each authentic pure enantiomer of ketorolac was spiked into drug-free plasma and subjected to the complete sample preparation method prior to chromatography to establish its retention time. Potential racemization during the derivatization procedure was also checked by this method.

Application of the Method

A healthy male volunteer (30 years) was administered a single 30 mg intramuscular dose of ketorolac tromethamine (Toradol®, Syntex) and post-dose blood samples were taken half hourly for the first 2 h then hourly for the next 6 h. Blood was sampled via an indwelling venous cannula sited in a forearm vein and immediately placed on ice (heparinized tubes) upon collection. Plasma was harvested (2,000*g*, 10 min) and 2 *M* sulphuric acid added (75 µl/ml of plasma) to prevent hydrolysis of potentially labile metabolic glucuronoconjugates[19] of ketorolac. Acid-quenched plasma was stored at −20°C prior to analysis. Aliquots (1 ml) of postdose plasma were analyzed to determine (R)- and (S)-ketorolac concentration (in terms of protein bound plus unbound enantiomer) as a function of time.

RESULTS AND DISCUSSION

Chromatograms obtained from the analysis of plasma sampled 3 h after a single 30 mg intramuscular dose of (RS)-ketorolac together with predose plasma spiked with 1600 ng/ml of racemic drug and internal standard alone are depicted in Figure 2. The retention times for (S)- and (R)-ketorolac, eluted as their respective diastereomeric (S)-1-phenylethylamide, were 6.5 and 7.2 min, respectively. (S)-Ketoprofen-(S)-1-phenylethylamide (internal standard) eluted at 8.6 min. The peaks of interest were adequately resolved and free of interfering endogenous plasma-derived signals.

Calibration curves generated over the enantiomeric concentration range of 50–3,200 ng/ml (supplied as racemic drug) were linear for both enantiomers of ketorolac. Linear least-squares regression analysis of 10 sets of calibration curves is given in Table 1. In each case the correlation coefficient was >0.999. In addition, assessment of the assay linearity was made by calculating the coefficient of variation (CV) derived from the mean enantiomer concentration–normalized peak area ratio (enantiomer to internal standard) for each series of calibration standards. Over the 4-week period during which 10 sets of plasma standards (50–3,200 ng/ml) were processed, the mean ± SD coefficients of variation for (S)-ketorolac and (R)-ketorolac were 5.67 ± 1.89 and 5.95 ± 2.12%, respectively.

The intraday accuracy and precision of the assay was assessed by analyzing four separate aliquots of three independently prepared racemic plasma standards containing 75, 200, and 1000 ng/ml of each enantiomer. For the quality control sample containing 75 ng/ml of each enantiomer, the mean concentrations of (S)- and (R)-ketorolac were determined to be 68.1 ng/ml (CV = 7.40%) and 72.2 ng/ml (CV = 8.73%), respectively. Analysis of the plasma sample containing 200 ng/ml of each enantiomer yielded predictions of 192 ng/ml (CV = 0.74%) and 198 ng/ml (CV = 1.12%) for (S)- and (R)-ketorolac, respectively. The predicted values for the plasma sample containing 1000 ng/ml of each enantiomer were 993 ng/ml (CV = 1.59%) for (S)-ketorolac and 1019 ng/ml (CV = 1.54%) for (R)-ketorolac.

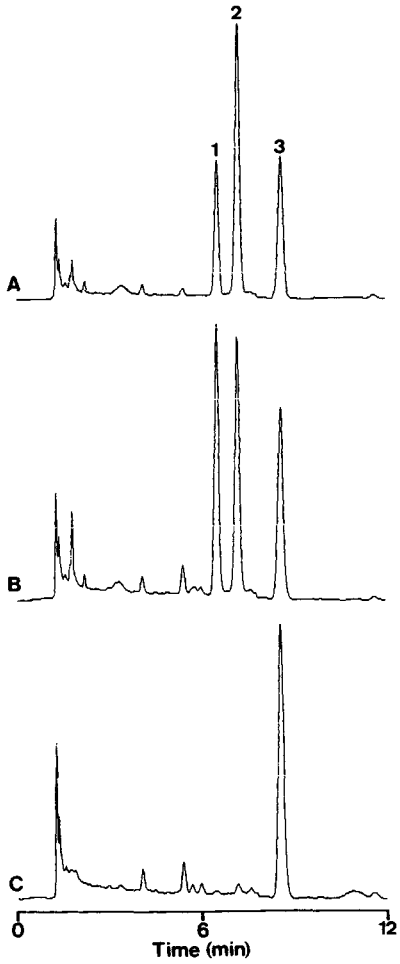

Fig. 2. Chromatograms derived from **(A)** plasma obtained 3.0 h after a 30 mg intramuscular dose of racemic ketorolac (Toradol®, Syntex) containing 560 ng/ml of (S)-ketorolac and 1,280 ng/ml of (R)-ketorolac; **(B)** drug-free plasma spiked with 1,600 ng/ml of (RS)-ketorolac; and **(C)** drug-free plasma spiked with internal standard alone. Peaks 1 and 2 are derivatized (S)- and (R)-ketorolac, respectively, and peak 3 is the derivatized internal standard ((S)-ketoprofen).

The interday accuracy and precision of the analytical method were determined by assaying the quality control plasma samples containing 75, 200, and 1000 ng/ml of each enantiomer [supplied as (RS)-ketorolac] over a 4-week period. The predicted ketorolac enantiomer concentrations of the quality control samples are given in Table 2.

The mean ± SD extraction of underivatized ketorolac from acidified plasma *(vide supra)* was 80.1 ± 4.4% for 200 ng/ml of (RS)-ketorolac ($n=6$) and 78.2 ± 1.8% for plasma containing 2000 ng/ml of (RS)-ketorolac ($n=6$). The mean ± SD efficiency of the extraction of derivatized ketorolac diastereomeric amides (1000 ng of each diastereomer, $n=6$) at the second extraction step was 76.6 ± 3.8% and 77.2 ± 4.1% for (S)- and (R)-ketorolac-(S)-1-phenylethylamide, respectively.

Derivatization of (RS)-ketorolac to the corresponding diastereomeric amides appeared to be complete. There was no detectable ketorolac free acid in plasma samples spiked with a

TABLE 1. Calibration data derived from linear least-squares regression analysis for each ketorolac enantiomer performed independently, over a 4-week period

Regression parameter	Mean (±SD) value (n = 10) (S)-Ketorolac	(R)-Ketorolac
Slope	817 ± 50	820 ± 45
y-Intercept	9.96 ± 23.0	15.6 ± 21.0
Regression coefficient	0.9995 ± 0.0002	0.9994 ± 0.0002
Concentration range (ng/ml)	50-3200	50-3200

TABLE 2. Interday accuracy and precision of the analytical method for (S)- and (R)-ketorolac in plasma performed over a 4-week period (n = 10 for each predicted enantiomer concentration)

Quality control concentration (ng/ml)	Mean (%CV) predicted concentration (ng/ml) (S)-Ketorolac	(R)-Ketorolac
75.0	76.6 (10.6 %)	74.6 (8.49%)
200	200 (4.65%)	202 (4.93%)
1000	1012 (3.60%)	1041 (2.78%)

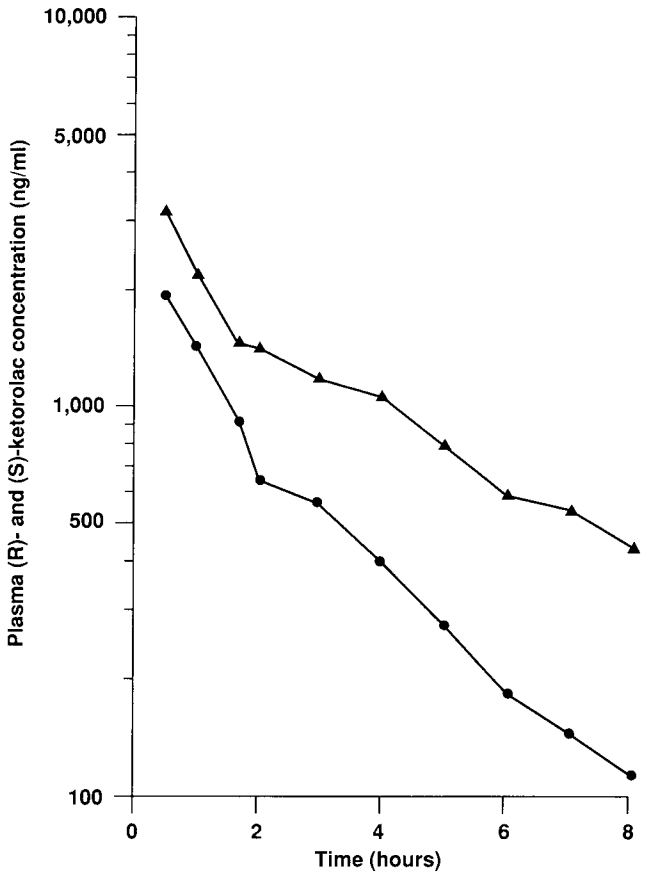

Fig. 3. Semilogarithmic plasma concentration-time profiles for (R)-ketorolac (▲) and (S)-ketorolac (●) from a human subject administered 30 mg of (RS)-ketorolac (as the tromethamine salt, Toradol®, Syntex) by intramuscular injection. Enantiomer concentrations are expressed in terms of the free acid.

range of ketorolac concentrations (100–6,400 ng/ml of racemic drug) and subjected to the derivatization conditions used herein. The derivatization procedure in this method was based on conditions employed for a structural congener (and the internal standard used in this assay).[16] Radiolabelled ketoprofen was synthesized[17] and used to show that generation of diastereomeric (S)-1-phenylethylamides of ketoprofen with thionyl chloride/(S)-1-phenylethylamine was nonstereoselective and quantitative.[16] The derivatization procedure for ketorolac did not lead to detectable racemization of either enantiomer. The concentration of the minor optical antipode was less than 3.0% when plasma samples spiked with either pure (R)- or (S)-ketorolac were assayed, stereoselectively. Moreover, there was no statistical difference (paired t test, $P > 0.05$) between the slopes of the linear regression lines for (R)- and (S)-ketorolac (Table 1) suggesting nonstereoselective diastereomeric amide formation.

In the course of development of this analytical method for ketorolac a number of chiral 2-aryl propionic acids were investigated as potential internal standards. The retention time of (R)-ketoprofen was 9.9 min, free of interfering peaks and resolved from its optical antipode. Indeed (RS)-ketoprofen (commercially available) could be used in lieu of (S)-ketoprofen as the internal standard. The retention times for the diastereomeric (S)-1-phenylethylamides of fenoprofen were 13.0 and 15.1 min, for ibuprofen 14.5 and 16.7 min, and for tiaprofenic acid 7.8 and 9.0 min, respectively.

The specificity of the assay procedure was investigated by derivatizing and injecting extracts of plasma samples from a number of patients who had been prescribed a variety of common drugs. Neither the parent drug nor any observed metabolite peak from a range of xenobiotics including methylprednisolone, verapamil, theophylline, fluoxetine, ranitidine, ethinyloestradiol, levonorgestrel, morphine, diazepam, and midazolam coeluted with either of the ketorolac diastereomers or the internal standard.

This analytical method for ketorolac enantiomers has been applied to a pharmacokinetic study in humans. The enantioselective disposition of ketorolac in a volunteer administered a 30 mg intramuscular dose of the tromethamine salt of (RS)-ketorolac (Toradol®, Syntex) is shown in Figure 3. The importance of using stereoselective analytical methods for ketorolac is self-evident from the plasma concentration-time profiles for each enantiomer. The plasma concentrations of the pharmacologically active (S)-enantiomer were significantly less than the corresponding concentrations of (R)-ketorolac at all time points examined. In addition, the elimination half-life of (S)-ketorolac in this subject was 2.2 h compared to 3.3 h for the (R)-enantiomer of ketorolac. Studies to elucidate the enantioselective pharmacokinetics and pharmacodynamics of ketorolac in humans are continuing in our laboratory.

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