The Problem of Racemization in the Stereospecific Assay and Pharmacokinetic Evaluation of Ketorolac in Human and Rats

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Purpose. A comparison of a previously reported indirect (precolumn derivatization) assay for ketorolac (KT) and a new direct method described here was made to establish the conditions under which KT may undergo racemization and to explain the observed discrepancies in the pharmacokinetics of KT reported in the literature.

Methods. A previously reported pre-column derivatization method and a new direct method were employed to determine the effect of pH and ionic strength on racemization. Using the conditions where no racemization occurred, the pharmacokinetics in humans and rats, and protein binding of KT enantiomers were determined.

Results. Under the chromatographic conditions employed for the direct assay, no racemization was observed. Under high pH and ionic strength, however, both methods resulted in KT racemization. The indirect method resulted in rapid and complete racemization due to the strong basic conditions required for derivatization. In both humans and rats, the pharmacokinetics of racemic KT were stereoselective with the R enantiomer being predominant (AUC S/R: humans, 0.26; Rats, 0.45). This is likely due to more extensive plasma protein binding of S than its antipode (unbound S/R: 1.35).

Conclusions. The discrepancies in the literature may be explained by rapid racemization of KT that occurs during sample preparation for the pre-column derivatization method. Considerations should be given to the possibility of racemization during the assay development and validation.

Key Words: ketorolac; racemization; inversion; stereospecific assay; pharmacokinetics.

INTRODUCTION

Ketorolac (KT) (Fig. 1) is a chiral nonsteroidal anti-inflammatory drug (NSAID) marketed as the racemate. Throughout the last 10 years, a growing awareness of the importance of chirality in the kinetics of NSAIDs has led to development of chiral assays for determination of stereoselective disposition of these drugs (1). However, many of the methods employed to assess the validity of such assays have been lacking data pertaining to racemization and/or stereoselectivity in sample preparation (e.g., extraction, derivatization). This was, in part, due to the novelty of the field, and in part, to the lack of availability of optically pure enantiomers as in the case of KT.

Recent advances in chiral separation technologies have made available more efficient chiral columns as well as stereochemically pure enantiomers. This has led to an explosion of convenient, sensitive direct chiral assays for many agents. The availability of pure enantiomers has also made possible proper methods for determining the validity of these methods, as well as the ability to determine possible racemization reactions occurring during the assay procedure.

Earlier reports indicated that in both humans (2) and rats (3) the plasma concentration time courses of KT enantiomers were superimposable. Recently, however, a number of authors using various assays have found stereoselectivity in the pharmacokinetics of KT enantiomers in human plasma (4-7). These findings are contradictory and raise the possibility of assay racemization. The purpose of this study was to explain these discrepancies and their underlying reasons, by comparing the earlier pre-column derivatization method (3) with a new direct HPLC assay, and delineate pharmacokinetics of KT in both humans and rats.

MATERIALS AND METHODS

Racemic KT was supplied by Syntex (Palo Alto, Ca). Individual Enantiomers (stereochemical purity <99.7%) were kindly supplied by Sepacol (Marlborough, MA). Naproxen was purchased from Sigma Chemical Company (St. Louis, Mo.). All Chemicals and solvents used were of analytical grade.

Standard Solutions and Calibration Curves for Direct Method

The internal standard (IS) solution was prepared by dissolving 10 mg naproxen in 100 mL of methanol. A stock solution of 10 mg racemic KT in 100 mL was also prepared in the same manner. Subsequent 1/10 and 1/100 dilutions of the stock solution of KT were made in methanol. Appropriate volumes of the above solutions were added to 0.5 mL of plasma to yield final enantiomer concentrations of 25, 50, 100, 500, 1000, and 2000 ng/mL used in the calibration curves.

Sample Preparation for Direct Method

Following addition of 0.05 mL of IS (100 μg/mL) and acidification with 0.2 mL of 0.6 M H2SO4, the constituents of 0.5 mL of human plasma or 0.1 mL of rat plasma were extracted with 3 mL of diethyl ether after vortex mixing for 30 seconds and centrifugation at 2500 rpm for 5 minutes (Adams Dynac Centrifuge: Clay-Adams, New York). The organic layer was then transferred to clean tubes and evaporated to dryness (Savant Speed Vac Concentrator/Evaporator: Emerson Instruments, Scarborough, Ontario, Canada). The remaining residue was dissolved in 0.2 mL of HPLC mobile phase and aliquots of 0.01 to 0.12 mL were injected into the HPLC.

Chromatographic Conditions

The HPLC used in this assay consisted of a SIL-9A autoinjector, a SPD-10A UV detector set to 313 nm, Shimadzu Ezchrom integration Software (Fisher Scientific, Ed-
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Fig. 1. Chemical structure of ketorolac.

monton, Alberta, Canada), and a model 590 pump (Waters Scientific, Mississauga, Ontario, Canada). Separation of KT enantiomers was performed using a guard column of silica (5 μm by 5 cm) attached to a 12.5 cm Partisil 5 ODS 3 column followed by a 5 cm chiral tetr-leucine column (all columns from Phenomenex, Torrance, CA). Mobile phase consisted of 0.5 mM ammonium acetate in a mixture of methanol/ethyl acetate/isopropanol (50:50:2 V/V) at ambient temperature, and a flow rate of 0.8 mL/min. Upon completion of this study we tested a new column (Chiralpak AD, Extion, PA) with a mobile phase consisting of hexane/isopropanol/trifluoroacetic acid (90:10:0.1 V/V).

Recovery from the Direct Method

The recovery of S and R-KT and JS was determined in triplicate at concentrations of 100 and 1000 ng/mL of each enantiomer. Extraction efficiency as percent yield was calculated by the ratio of area of each enantiomer following direct injection of the methanol stock solution over that following extraction using the above mentioned extraction procedure.

Precision and Accuracy of the Direct Method

Precision was calculated by intra and interday coefficients of variation of the calibration curve slopes. Accuracy was calculated from blinded unknowns at both high (1000 ng/mL) and low (25 ng/mL) concentrations. All experiments were performed in triplicate for 3 consecutive days.

Racemization Study

The extent of assay racemization was studied for the previously reported indirect method (3) involving ethylchloroformate and L-leucinamide as derivatizing reagents by spiking aliquots of 0.5 mL of plasma with 100 μL of a 10 μg/mL solution of S-KT and assaying. The effect of a 24 h incubation, pH and ionic strength on the extent of racemization of the enantiomers and racemic KT was investigated using the direct method. Buffer solutions of pH 1 (0.2N KCl/HCl), 3 (0.1M glycine/NaCl/HCl), 5, 7 (0.075M mono/0.015 disodium phosphate buffer), 9, 11, and 13 (0.1N glycine/0.1N NaCl/NaOH) were prepared. To 0.5 mL of each of these buffers was added 100 μL of a 10 μg/mL solution of S-KT. The samples were acidified, and assayed using the process described herein. The effect of ionic strength on racemization of KT was examined. To a buffer solution (pH 13) was added sodium chloride to yield ionic strengths (μ) of 0.1, 0.5, 1, and 2. In addition, the racemization of KT was also tested in a pH 7 buffer solution with ionic strength of 2. To 0.5 mL of each of these solutions was added 100 μL of a 10 μg/mL solution of S-KT. The samples were acidified and assayed using the present method.

Pharmacokinetic Studies

Adult male Sprague Dawley rats weighing between 300 and 400 g were used to determine the kinetics of KT enantiomers in the rat. Catheterization of rats at the right jugular vein was performed using sialastic tubing (0.58 mm i.d. x 0.965 mm o.d.; Clay Adams, Parsippany, NJ) Animals were allowed to recover overnight and had access to water ad libitum. They were fasted overnight until 3 h post dose. Racemic KT (1 mg/kg) dissolved in polyethylene glycol 400 was administered orally to each of the animals at 9 am on the day following surgery. Blood (0.2ml) was collected from the jugular vein cannula at 0.0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 24, h after KT administration. The catheter was flushed with 0.2 mL of 100 U/mL heparin following each blood sample collection. Immediately following collection, plasma was separated from blood by centrifugation at 1800 g for 3 min using a Fisher model 235A microcentrifuge (Fisher Scientific, Edmonton, Canada). All samples were stored at -20°C until analyzed.

The human study was conducted in accordance with the declaration of Helsinki. Three healthy male volunteers each took one 10 mg racemic KT (Syntex, Mississauga, Canada) at 9 am following overnight fasting. Venous blood samples (6-8ml) were collected at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, and 24 h. All samples were immediately centrifuged and the plasma was separated. To avoid possible cleavage of conjugates and possible racemization, all samples were stored at -20°C in previously acid-rinsed containers until analyzed.

Protein Binding

Stereoselective binding of KT enantiomers in plasma was determined using an ultrafiltration technique. Human pooled plasma (1 ml) spiked with racemic KT at concentrations of 0.5, 1, 2, and 5 mg/L (n = 3) was centrifuged at 37°C for 15 min through Diaflo membranes attached to amicon ultrafiltration tubes (Micon Micropartition System, Amicon Div., W.R. Grace and Co. Danvers, MA, USA). Due to the large degree of plasma binding, samples at 0.5 mg/L concentrations, were pooled (5 cells per sample). The free fraction was calculated by dividing the total amount of each enantiomer in ultrafiltrate by the sum of amount in ultrafiltrate and amount remaining in plasma and multiplying by 100.

Data Analysis

\[ AUC_{0\rightarrow t} = AUC_{0\rightarrow \infty} + C / \beta \]

where t was the last sampling time and C, the last measured concentration. The rate constant of the log-linear terminal portion (β) was estimated using linear regression. The oral clearance (CL/F) was calculated using the enantiomeric dose divided by the respective AUC_{0\rightarrow t}. The apparent volume of distribution was calculated as \[ \text{Vd/F} = D / AUC_{0\rightarrow \infty} \cdot \beta \] where F is the fraction of the dose absorbed.

Statistical Analysis

All values are reported as mean ± standard deviation. The differences between enantiomers of KT pharmacokinetic indices were assessed using Student's two sided t-test at an α = 0.05 level of significance. Multiple group study de-