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Report

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 superimposible. 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 Sepracor (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 H_2SO_4, 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: Emerston 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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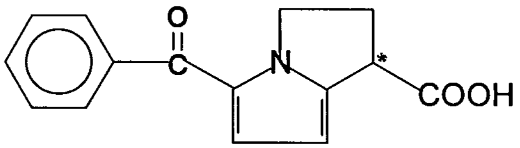

Fig. 1. Chemical structure of ketorolac.

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 tirt-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, Exton, PA) with a mobile phase consisting of hexane/isopropanol/trifluoro acetic acid (90:10:0.1 V/V).

Recovery from the Direct Method

The recovery of S and R-KT and IS 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 ug/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

$AUC_{0\text{-}t}$ was measured by the linear trapezoidal method. $AUC_{0\text{-}\infty} = AUC_{0\text{-}t} + C_t/\beta$ where t was the last sampling time and C_t 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 $AUC_{0\text{-}\infty}$. The apparent volume of distribution was calculated as $Vd/F = D / AUC_{0\text{-}\infty} \cdot \beta$ 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 $\alpha = 0.05$ level of significance. Multiple group study designs (effect of pH, ionic strength, extraction efficiency) were assessed using one-way ANOVA techniques at $\alpha = 0.05$ level of significance followed by Scheffe post-hoc analysis. The best fit through the calibration curve data was estimated from linear least-squares regression.

RESULTS

Using the direct method, the S and R enantiomers of KT and IS were resolved 12, 13 and 21 min post injection with a near baseline separation of the enantiomers (Fig.2). The resultant calibration curves were linear with correlation coefficient of >0.998. Typical regression lines were $Y = 1.42x + 0.0045$ and $Y = 1.40x + 0.0034$ for the S and R enantiomers, respectively. The extraction efficiency of the enantiomers and IS exceeded 80% with no stereoselectivity in the process. Limited coefficient of variations (CV) were observed in the calibration curve slopes (intraday: S, 4.76%; R, 3.91%, interday: S, 10.4%; R, 10.42%). The mean percent error of unknowns was below 9.5 and 1.8% for both enantiomers at 0.025 and 1 μg/mL concentrations, respectively. Complete baseline separation was achieved using a Chirapak AD column (data not shown).

Racemization of S and R-KT with the indirect method using triethylamine, ethylchloroformate and 1-leucinamide in acetonitrile was rapid and complete (S:R ratio of 0.97). In contrast, there was no enantiomeric conversion of either R or S enantiomers within 24 h under the chromatographic conditions described for the direct method. The S:R ratio remained constant when the racemate was subjected to both assays.

Using the direct method, addition of 100 μL of a 1M NaOH solution to 100 μL of a 1 μg/mL S-KT resulted in complete racemization. No chiral conversion was noted when stereochemically pure enantiomers were incubated in various buffer solutions with pH ranging from 1 to 11 (Fig. 3a). At pH 13, however, a 1.5% chiral conversion was observed. In addition, at pH 13, an increase in ionic strength significantly enhanced the extent of racemization (Fig. 3b). At pH 7, increased ionic strength did not result in racemization.

Marked stereoselectivity was observed in the plasma concentration vs. time profiles in rats (Fig. 4C, Table I) for the enantiomers of KT indicated by significant differences in $AUC_{0-\infty}$ (S/R Ratio 0.45), CL/F and C_{max}. There was a trend for a larger Vd/F for S-KT compared to R-KT ($p=0.08$). Both enantiomers of KT were rapidly eliminated with terminal $t_{1/2}$ values which were not significantly different from one another (Table I).

Significant stereoselectivity in the human plasma concentration vs. time profiles were also observed (Fig. 4A, Table I). There was significant differences between S and R-KT for $AUC_{0-\infty}$, CL/F, and C_{max} values. The S/R ratio of $AUC_{0-\infty}$ was 0.26 ± 0.10. There is also a trend ($p=0.08$) towards longer half life for the R enantiomer as compared with its antipode.

Both S and R-KT were extensively bound to plasma protein (99.39% and 99.55% respectively). Significant stereoselectivity in the free fraction was observed ($p < 0.0001$) with a mean S/R ratio of 1.35 ± 0.18. The plasma

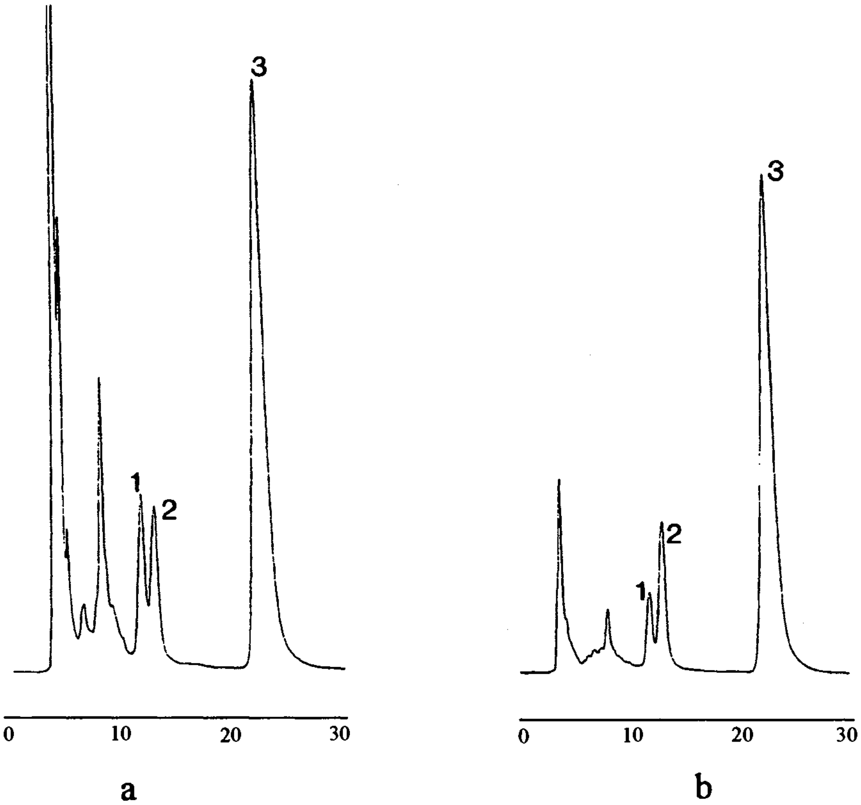

Fig. 2. Chromatograms for KT in plasma from direct method for 50 mg/L from calibration curve (a) 6 hour *in vivo* sample in a human volunteer (b) (minutes on x axis). Key 1, S-KT; 2, R-KT; 3, IS.

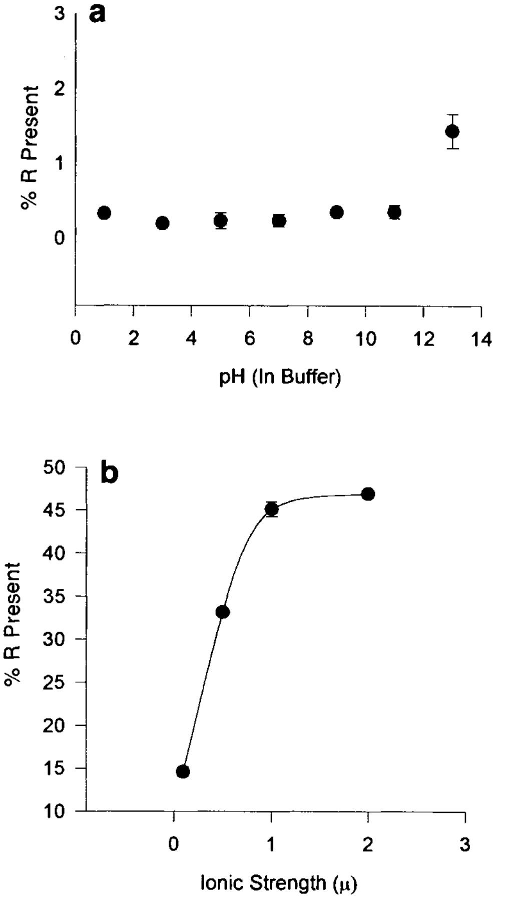

Fig. 3. Effect of pH on the %R-KT present (a) and ionic strength (μ) at pH 13 (b) following injection of S-KT.

protein binding was not concentration dependent within the examined concentration range (Fig. 5).

DISCUSSION

The enantiomers of KT undergo complete and rapid racemization using the previously reported derivatization method of analysis (3). The racemization during the sample preparation is likely due to the alkaline conditions imposed by the triethylamine (TEA) required for derivatization. Using the direct column resolution, no racemization was observed at various pH values up to 11. There was, however, a small but significant amount of racemization (1.5%) at pH 13, which increased to close to 100% with increasing ionic strength (Fig. 3). A plausible explanation would be enhanced stability of an intermediate, charged, species formed during the reaction at high ionic strength and pH. Despite a high ionic strength, KT was stereochemically stable at pH 7, suggesting that a high pH is a co-requisite for racemization. It has been suggested that the carboxy anion facilitates racemization (8). The molecular structure is also important in the configurational stability. For example, the presence of a tertiary nitrogen group may result in reduced configurational stability of the chiral carbon due to its acid strengthening properties (8). It has also been suggested that, for some other NSAIDs, the concentration of ethylchloroformate (ETC) used as a coupling reagent during the process of derivatization is an important factor for complete and/or partial isomerization: The lower the concentration of ETC, the higher the degree of enantiomeric conversion due, perhaps, to the predominance of the derivatization reaction as compared with the racemization pathway at the higher ETC concentration (9).

It is presumed that the carboxylic acid group is ionized in the aqueous medium. Under non-aqueous condition, on the other hand, reaction of KT with ETC/TEA, is expected to yield the mixed anhydride product. The proton on the chiral center is, therefore, rendered sufficiently acidic such that isomerization also occurs under these non-aqueous conditions.

Among the examined NSAIDs (9), KT undergoes the highest extent of racemization. This is likely due to the fact that the various factors (e.g., pH, ionic strength, presence of other compounds) involved in the process of racemization affect different NSAIDS to varying degrees. Hence, in addition to the experimental conditions, the degree of the configurational structure stability appears to be dependent upon the NSAIDs molecular structure. The conditions applied during the pre-column derivatization are optimal for racemization of KT, but not for other NSAIDs (9).

The observed chemical isomerization which occurs in high alkali medium, in the presence and absence of derivatization reagents, is different from chiral metabolic inversion, which is reported for some 2-arylpropionic acids (1), in that the former is bidirectional (S→R and R→S) while the latter is mainly, in all known cases, in the direction of R→S due to the enantioselective nature of the enzyme involved.

Both direct and indirect methods may involve sample preparation and derivatization processes which may render the molecule stereochemically unstable. For example, strong base reagents have been used to cleave β-glucuronidase resistant acylglucuronides of KT in urine. As we have shown, the use of strong base to cleave KT glucuronides for KT is not a suitable technique as it results in racemization. Diaz-Perez et al (7) who studied KT glucuronides in urine using a direct method after cleavage with a strong base, reported no stereoselectivity in the urinary excretion of the metabolite. Their observation, however, may well reflect racemization of the enantiomers under extreme basic condition.

The direct method presented herein is specific, convenient, and sensitive for stereospecific analysis of KT in plasma of both rats and humans. Chromatograms presented here are all generated using a tirt-leucine column. Although we had excellent reproducibility and accuracy, KT peaks did not have complete baseline separation. The use of a Chiralpak AD column that we tested upon completion of this study, on the other hand, resulted in baseline resolution. The sensitivity of the assay (25 ng/mL based on 0.5 mL human plasma sample) provided us with the opportunity to study protein binding of KT in human plasma spiked with therapeutically relevant concentrations of the enentiomers. The results of our protein binding study are in agreement with those recently reported using tritium labeled KT (10) at concentrations approximately four fold greater than those used in this study. This further supports our observation (Fig. 5) that protein binding of KT enantiomers is concentration independent.

After administration of racemic KT, the pharmacokinet-

Table I. Pharmacokinetic Indices of KT Enantiomers Following 1 mg/kg or 10 mg Single Oral Racemic Doses to Male Sprague Dawley Rats or Healthy Human Volunteers

	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0-\infty}$ (ng h/mL)	$t_{1/2}$ (h)	Vd/F (L/kg)	CL/F (L/h/kg)
Rats (n = 3)						
S	0.50 ± 0.0	588 ± 504[a]	681 ± 104[a]	1.50 ± 0.68	1.55 ± 0.44	0.75 ± 0.12[a]
R	0.50 ± 0.0	984 ± 507[a]	1525 ± 88[a]	0.93 ± 0.44	0.44 ± 0.22	0.32 ± 0.02[a]
Humans (n = 3)						
S	1.08 ± 0.80	210 ± 77[a]	708 ± 233[a]	1.24 ± 0.33	0.167 ± 0.10	0.091 ± 0.036[a]
R	1.03 ± 0.80	464 ± 195[a]	2742 ± 306[a]	2.44 ± 0.84	0.076 ± 0.02	0.022 ± 0.002[a]

[a] Significantly different than its antipode.

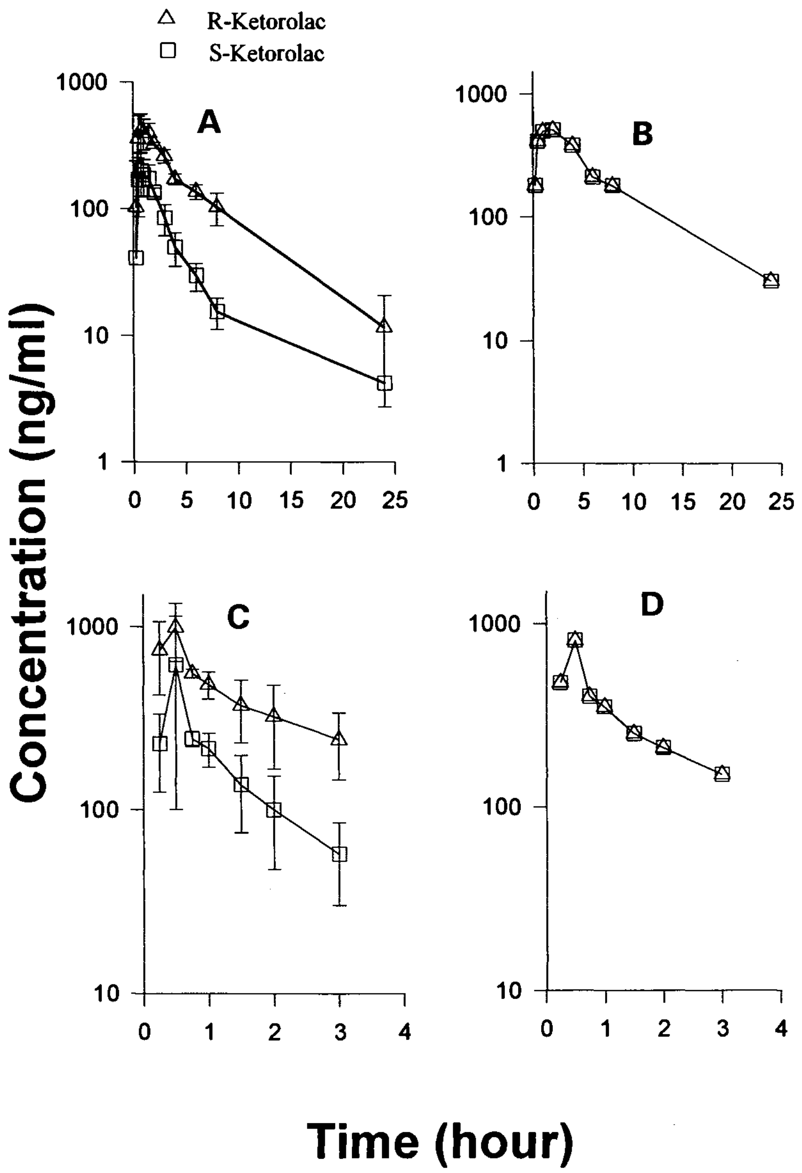

Fig. 4. Mean plasma concentration vs. time profiles of KT enantiomers following oral dosing of 10 mg of racemic KT to human volunteers (A), 1 mg/kg of racemate to male Sprague-Dawley rats (C), concentration-time profiles reported in the literature (references 2 and 3 with permission) for humans (B) and rats (D) using indirect precolumn derivatization method. Error bars indicate standard deviation.

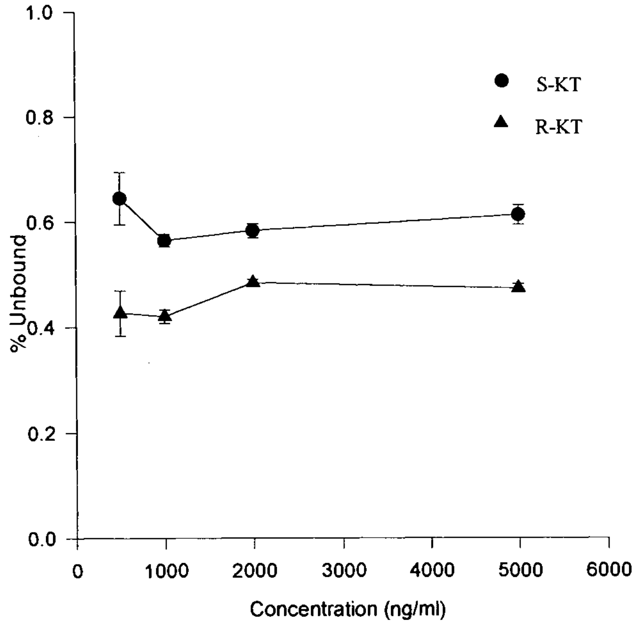

Fig. 5. Mean % unbound vs concentration of KT in pooled human plasma.

ics of KT show marked stereoselectivity in humans (Fig. 4A). Plasma concentrations of R-KT were significantly higher than those of S-KT. These results which support more recent reports (4-7) are in contrast with an observation previously made in a single subject using a precolumn derivatization HPLC method (Fig 4b) (2). The observed stereoselectivity may be explained, at least in part, by the higher unbound fraction of S-KT in plasma (Fig. 5). This however, does not exclude other mechanisms such as stereoselective inversion metabolism or tissue binding. It is interesting to note that, similar to etodolac (11), but in contrast to the majority of other chiral NSAIDs, the pharmacologically less active R enantiomer is predominant in plasma. This may exclude the possibility of a appreciable unidirectional metabolic R to S inversion which is reported for some other 2-arylpropionic NSAIDs (1). In a previous paper, the enantiomers of KT were reported to exhibit superimposible plasma concentration-time courses following administration of racemic KT to the rat (Fig. 4D) (3). In this study, however, we observed marked stereoselectivity in the pharmacokinetics of KT in male Sprague Dawley rats (Fig 4C). The discrepancy between the two studies is likely due to the problem of racemization associated with the previous method used (3). The plasma concentration profiles of KT enantiomers in the rat were qualitatively similar to those observed in humans, with plasma concentrations of R being greater than those of S-KT. This may make the rat a suitable animal model for pharmacokinetic studies of the drug.

The study of chiral agents has brought with it a number of unique considerations for assay development. Inappropriate or incomplete validation using only the racemate may lead to discrepancies in findings. Chiral assays should include studies carried out to ensure the stereochemical stability of the enantiomers under various conditions. When available, stereochemically pure enantiomers should be studied for the possibility of chemical conversion or racemization. When stereochemically pure enantiomers are unavailable, as has been the case in the past with many drugs including KT, separation and purification may be achieved by fraction collection after column separation or in vivo enrichment (9).

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