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The Direct Determination of the Enantiomers of Ketorolac and *para*hydroxyketorolac in Plasma and Urine Using Enantioselective Liquid Chromatography on a Human Serum Albumin-Based Chiral Stationary Phase

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ABSTRACT An enantioselective assay has been developed for the determination of the enantiomers of ketorolac and its metabolite *p*-hydroxyketorolac in plasma and urine. The analytical method utilizes a coupled achiral–chiral HPLC system where the initial separation of ketorolac from *p*-hydroxyketorolac and matrix interferences was achieved on a C_{18}-stationary phase and the enantioselective separations of the two target solutes were accomplished on a human serum albumin-based chiral stationary phase. The two columns were attached in sequence and the assay was carried out without the necessity of column-switching techniques. The method has been validated for use in pharmacokinetic and metabolic studies and represents the initial report of the determination of ketorolac and *p*-hydroxyketorolac enantiomers in urine. The results of the study indicate that after the administration of racemic ketorolac there was an enantioselective distribution of ketorolac enantiomers in plasma [(R)-ketorolac: (S)-ketorolac = 3.89 ± 0.93 (n = 6) and urine (R)-ketorolac: (S)-ketorolac = 1.26 ± 0.09 (n = 7)]. The mean ratio of the *p*-hydroxyketorolac enantiomers was 1.77 ± 0.46 (n = 7). Both ketorolac and *p*-hydroxyketorolac are glucuronized in the acyl carboxyl moiety and the results of this study indicate that this process is not enantiospecific.

Ketorolac [(±)-5 benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid, Ket, Fig. 1] is an analgesic and nonsteroidal antiinflammatory drug (NSAID) which inhibits the arachidonic acid cascade at the cyclooxygenase level.[1] It displays nonirritating ophthalmic antiinflammatory activity and it has been used with success in the treatment of postoperative cystoid macular edema, a frequent cause of decreased vision after cataract extraction.[2]

Ket is a chiral compound which is marketed as the racemic mixture. Previous animal studies have shown that the (S)-enantiomer is more potent than the (R)-enantiomer of Ket.[3] The pharmacokinetics profiles of the Ket enantiomers has also been studied in humans and (S)-Ket has a higher clearance (CL) and a greater volume of distribution (V_{SS}) than (R)-Ket, while (R)-Ket has a longer elimination half-life.[4] The major metabolic route of Ket in humans is acyl glucuronidation which accounts for 28% of the administered dose and a second pathway accounting for 12% of the administered dose is *p*-hydroxylation, which produces p-OH-Ket.[5] To our knowledge the enantioselectivity of the urinary excretion of Ket and *p*-OH-Ket in humans has not been determined.

The enantioselective disposition of Ket demonstrated the necessity of using enantioselective analytical methods. Earlier human and animal HPLC studies have been reported for the analysis of Ket in biological fliuds, however, most did not discriminate between the Ket enantiomers.[6–8] An enantioselective assay for (S)-Ket and (R)-Ket in human plasma has been reported.[9] This method involved derivatization of Ket with (S)-1-phenylethylamine followed by stereochemical resolution of the diastereomers using reversed-phase chromatography.

In order to quantitate the enantiomers of Ket and *p*-OH-Ket in plasma and urine, we have developed and validated a sensitive and specific HPLC method for use after administration of therapeutic doses of the racemic drug. The assay utilizes a chiral stationary phase based on immobilized human serum albumin (HSA-CSP) which has been previously shown to enantioselectively resolve other NSAIDs such as suprofen, ketoprofen, and benoxaprofen. The enantiomers of Ket and *p*-OH-Ket were separated using a coupled achiral/chiral HPLC system where an achiral C_{18} column was coupled on-line to the HSA-CSP column. The assay was applied to plasma and urine from patients undergoing cataract surgery and the results indicated that the plasma and urine concentrations of (R)-Ket were greater than (S)-Ket and that the urinary excre-

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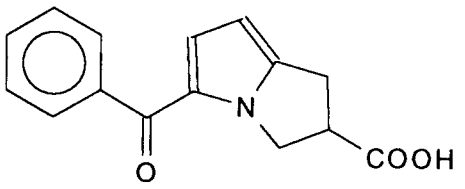
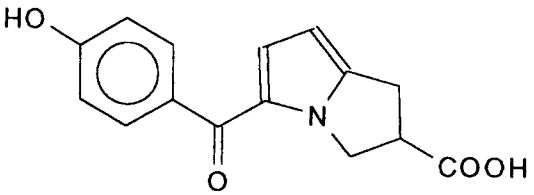
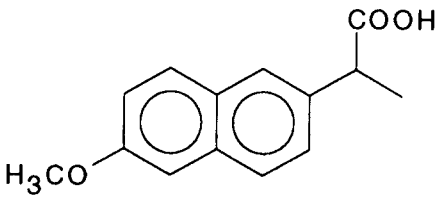


KETOROLAC



p-OH-KETOROLAC



S-NAPROXEN

Fig. 1. Chemical structures of the compounds analyzed in the study.

tion of *p*-OH-Ket was also enantioselective. In addition the acyl glucuronidation did not appear to be enantioselective.

MATERIALS AND METHODS

Chemicals

Racemic ketorolac (*rac*-Ket), its separated enantiomers [(R)-Ket and (S)-Ket], racemic *p*-OH-ketorolac (*rac-p*-OH-Ket), and (S)-naproxen [(S)-Nap] were provided by Syntex Research (Palo Alto, CA). Acetonitrile, hexane, and ethylacetate were HPLC grade (Anachemia, Montreal, Canada) and octanoic acid was of reagent grade (Aldrich, Milwaukee, WI).

High-Performance Liquid Chromatography

Apparatus The liquid chromatograph consisted of a Model P4000 pump, AS3000 autosampler, and SP4600 integrator all from Spectra-Physics (San Jose, CA), and a programmable Model 783 UV absorption detector from ABI (Ramsey, NJ).

Separations were performed using on-line coupling of an achiral analytical column, Merck LiChrospher 100 RP-18 (125 × 4 mm i.d.; particle size 5 μm; E. Merk, Darmstadt, Germany) and a chiral analytical column (HSA-CSP) based on immobilised human serum albumin (150 × 4.6 mm i.d.; particle size 5 μm; chiral protein column HSA, Shandon HPLC, Runcorn, UK), together with a Regis C_{18} guard column (10 × 3 mm i.d.; particle size 3 μm; Regis Chemical Company, Morton Grove, IL).

Chromatographic conditions The mobile phase was sodium dihydrogen phosphate–disodium hydrogen phosphate (0.05 *M*, pH 6.9), modified with 13% (v/v) acetonitrile. Octanoic acid was added to the mobile phase to produce a 0.1 m*M* concentration of the octanoic acid. Chromatography was performed at ambient temperature and using a flow rate of 1 ml/min. Detection of the peaks of interest was accomplished using programmable UV detection set at 313 nm for Ket and *p*-OH-Ket enantiomers, and then changed to 265 nm for detection of the internal standard [(S)-Nap].

The elution order of (S)-Ket and (R)-Ket was determined by chromatographing unequal mixtures of the two enantiomers. Since the separate enantiomers of *p*-OH-Ket were not available, the eluting peaks were labeled *p*-OH-Ket_1 and

TABLE 1. Chromatographic results

Compound	Retention time (min)	Capacity factor (k')	Enantioselectivity (α)	Enantioselective resolution factor (R_s)
HSA				
p-OH-Ket_1	4.88	1.68	2.02	2.73
p-OH-Ket_2	8.17	3.40		
(S)-Ket	6.32	2.43	4.19	8.00
(R)-Ket	20.85	10.18		
C_{18}-HSA				
p-OH-Ket_1	8.11	1.57	1.74	2.57
p-OH-Ket_2	11.88	2.73		
(S)-Ket	17.10	4.38	2.09	7.40
(R)-Ket	32.51	9.15		

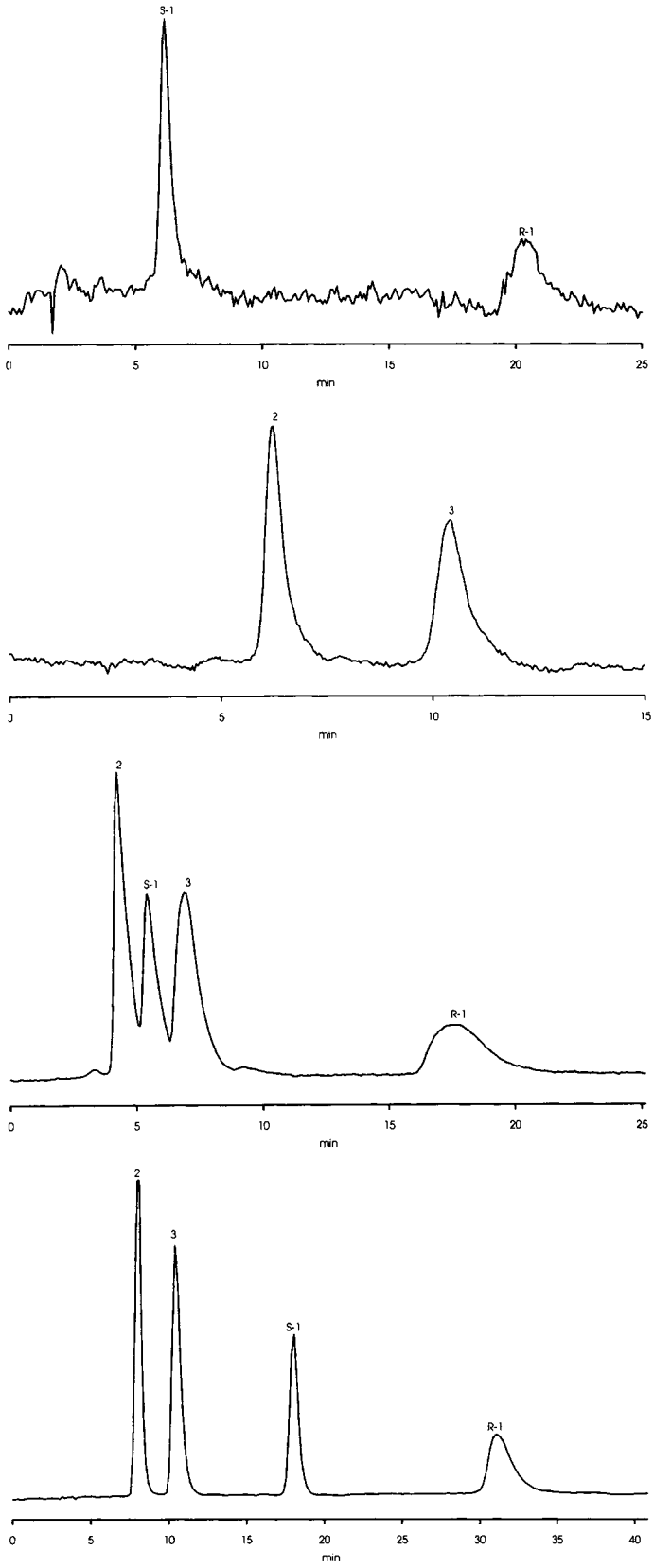


Fig. 2. Enantioselective separation of ketorolac and *p*-OH-ketorolac on HSA-CSP and coupled C_{18}-HSA-CSP. **(A)** Ketorolac on HSA-CSP; **(B)** *p*-OH-ketorolac on HSA-CSP; **(C)** ketorolac and *p*-OH-ketorolac on HSA-CPS; **(D)** ketorolac and *p*-OH-ketorolac on C_{18}-HSA-CSP. S-1, (S)-ketorolac; R-1, (R)-ketorolac; 2, *p*-OH-ketorolac$_1$; 3, *p*-OH-ketorolac$_2$.

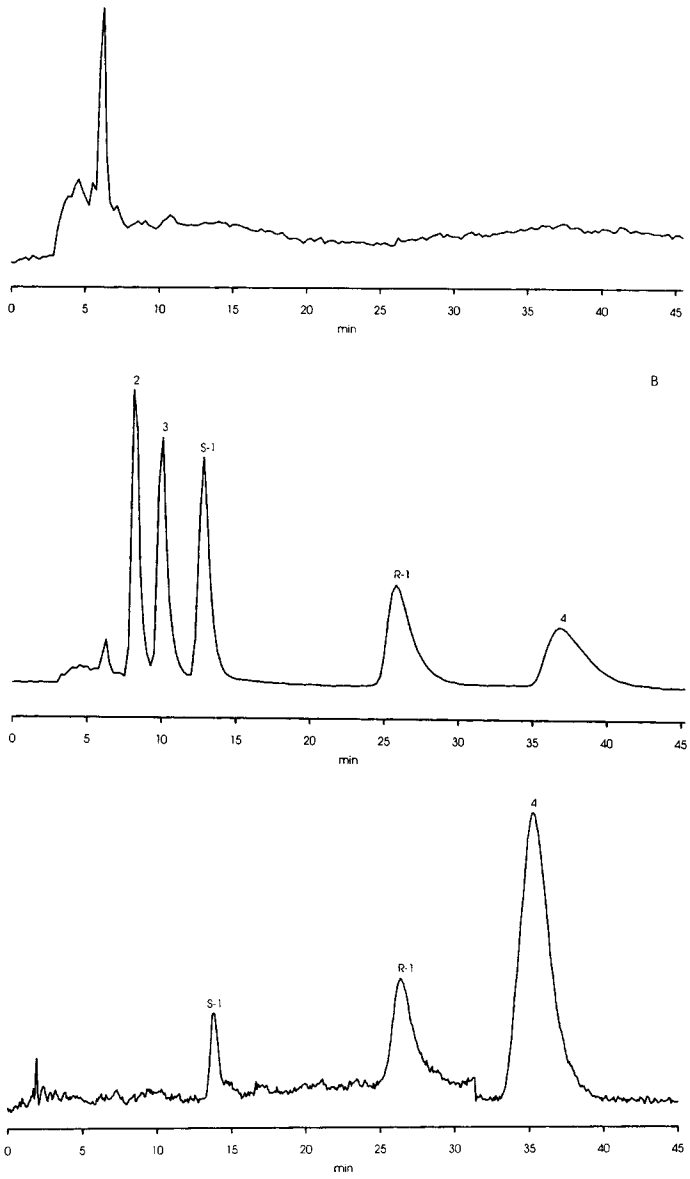


Fig. 3. Chromatograms of blank plasma (**A**), blank plasma spiked with 1.2 μg/ml of *rac*-ketorolac and *rac*-*p*-OH-ketorolac and 5 μg/ml of S-naproxen (**B**), and a 3 h plasma sample taken from a patient who received 10 mg of oral *rac*-ketorolac (**C**). S-1, (S)-ketorolac; R-1, (R)-ketorolac; 2, *p*-OH-ketorolac$_1$; 3, *p*-OH-ketorolac$_2$; 4, S-naproxen.

p-OH-Ket$_2$ to describe their order of elution from the HSA-CSP.

Sample Preparation

Stock solutions of *rac*-Ket at a concentration of 4 mg/ml, *rac*-p-OH-Ket at 1 mg/ml, and (S)-Nap at 100 and 50 μg/ml were prepared in methanol and used to spike plasma and urine for the analytical study.

To 1 ml of human plasma or urine were added 100 μl of one of the internal standard solutions (50 μg/ml for plasma or 100 μg/ml for urine), 300 μl of 1 *N* hydrochloric acid, and 2 ml of 30% ethylacetate in hexane. The mixture was vortex mixed for 2 min, centrifuged at 1500*g*, for 10 min, the aqueous layer frozen in an acetone/dry ice bath, and the organic layer transferred to a clean tube. The extraction procedure was repeated and the organic layers combined and evaporated to dryness under nitrogen. The residue was reconstituted in 250 μl (plasma) or 1000 μl (urine) of mobile phase and 20 μl were injected onto the HPLC system.

The ester conjugates of Ket and *p*-OH-Ket in the urine samples were hydrolyzed by addition of 200 μl 1 *N* NaOH at room temperature, and vortex mixing during 1 min. This

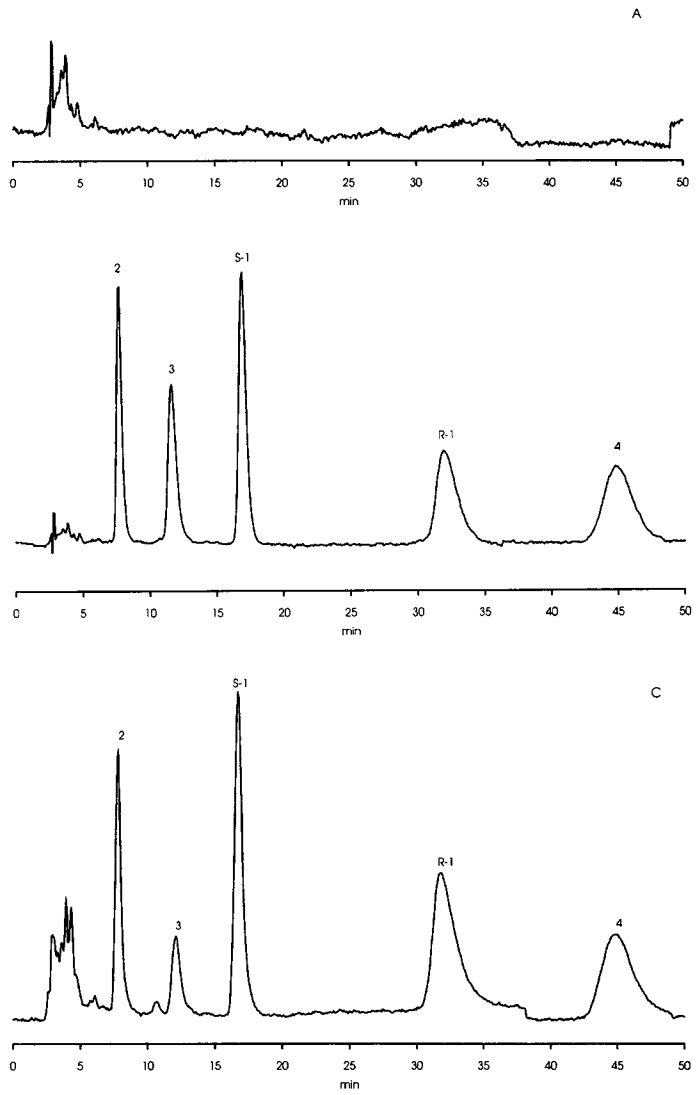


Fig. 4. Chromatograms of blank urine **(A)**, blank urine spiked with 1.5 μg/ml of *rac*-ketorolac, 2.5 μg/ml of *rac*-*p*-OH-ketorolac, and 10 μg/ml of (S)-naproxen **(B)**, and a nondeconjugated urine sample taken from a patient who received 10 mg of oral *rac*-ketorolac **(C)**. S-1, (S)-ketorolac; R-1, (R)-ketorolac; 2, *p*-OH-ketorolac$_1$, 3, *p*-OH-ketorolac$_2$; 4, S-naproxen.

method has previously been utilized for hydrolysis of ester conjugates of other nonsteroidal antiinflammatory drugs (NSAIDs) such as etodolac.[10] The samples were then acidified by addition of 300 μl 1 *N* HCl and extracted in the same way as described above. The difference between the peaks before and after hydrolysis was considered as the concentration of the conjugated drug in the sample.

Calibration Curve

Plasma standards were prepared by spiking blank plasma with *rac*-Ket and *rac*-*p*-OH-Ket to achieve final concentrations of 100, 200, 400, 600, 800, 1000, and 1200 ng/ml for each enantiomer. Urine standard samples were prepared by spiking blank urine with *rac*-Ket and *rac*-*p*-OH-Ket to achieve final concentrations of 250, 500, 2500, 5000, 7500, and 10,000 ng/ml for each *p*-OH-Ket enantiomer, and 500, 2500, 5000, 7500, 12,500, and 17,500 ng/ml for each Ket enantiomer. These samples were processed in the manner described above. Separate calibration curves for each enantiomer of Ket and *p*-OH-Ket were constructed using peak area ratios of the enantiomers to the internal standard and linear least-squares regression analysis.

Precision and Accuracy

The precision of the method was calculated based on the intra and interday coefficients of variance, CVs. The concentrations for each enantiomers of Ket and *p*-OH-Ket were 100 and 1200 ng/ml in plasma, whereas in urine the concentrations were 250, 2500, and 7500 ng/ml for each enantiomer of *p*-OH-Ket, and 500, 5000, and 12,500 ng/ml for each enantiomer of Ket. All samples were extracted in the manner described above. The intraday CVs were determined from data gener-

ated after repeated injections ($n = 10$) of the standard concentrations. To calculate the interday CVs triplicated injections of the standard concentrations were done during 4 days.

The accuracy of the method was determined using blinded unknowns prepared by a second analyst. In plasma the concentrations spiked were 100 and 200 ng/ml for each enantiomer of *p*-OH-Ket, and 100 and 700 ng/ml for each Ket enantiomer. The concentrations spiked in urine were 600, 4000, and 8000 ng/ml for each *p*-OH-Ket enantiomer, and 800, 2000, and 16,000 ng/ml for each Ket enantiomer.

Duplicate plasma and urine samples were analyzed using the method described above and the accuracy was determined by comparing the estimated concentrations with the known concentrations of the analytes.

Recoveries

The recoveries of the enantiomers of Ket and its metabolite were determined in triplicate at concentrations within the range of the calibration curves. The concentrations for each enantiomer of *p*-OH-Ket and Ket in plasma were 400 and 1000 ng/ml. The urine concentrations for each enantiomer of *p*-OH-Ket and Ket were 1000 and 10,000 ng/ml. Recoveries were measured by contrasting the areas of the extracted samples with those obtained after injection of unextracted samples. The extraction efficiency of the (S)-Nap was also determined at concentrations of 5 μg/ml in plasma and 10 μg/ml in urine.

Clinical Study

Patients undergoing cataract surgery received a 10 mg oral dose of *rac*-Ket three times per day for 2 days prior surgery. On the morning of the day of surgery, urine samples were collected and the patients were given a 10 mg oral dose of *rac*-Ket approximately 3 h prior surgery. At the time of cataract removal, blood, aqueous, and vitreous humor samples were taken; the blood samples were centrifuged and the plasma collected. All samples were stored at −80°C until assay.

TABLE 2. Intraday validation of ketorolac (Ket) and *p*-OH-Ket in plasma

Concentration (ng/ml)	Peak area ratio (mean ± SD, $n = 10$)	Coefficient of variation (%)
p-OH-Ket$_1$		
100	0.084 ± 0.002	2.73
1200	1.085 ± 0.045	4.10
p-OH-Ket$_2$		
100	0.086 ± 0.005	5.98
1200	1.144 ± 0.053	4.69
(S)-Ket		
100	0.079 ± 0.025	3.17
1200	1.040 ± 0.050	5.76
(R)-Ket		
100	0.077 ± 0.026	3.38
1200	1.070 ± 0.055	5.14

TABLE 3. Interday validation of Ketorolac (Ket) and *p*-OH-Ket in plasma

Concentration (ng/ml)	Peak area ratio (mean ± SD, $n = 12$)	Coefficient of variation (%)
p-OH-Ket$_1$		
100	0.083 ± 0.005	6.09
1200	1.050 ± 0.070	6.80
p-OH-Ket$_2$		
100	0.086 ± 0.007	8.02
1200	1.052 ± 0.076	7.30
(S)-Ket		
100	0.086 ± 0.007	8.30
1200	1.044 ± 0.091	8.73
(R)-Ket		
100	0.083 ± 0.008	10.10
1200	1.040 ± 0.090	8.80

RESULTS

In an attempt to develop an enantioselective assay to resolve the enantiomers of Ket and its metabolite, *p*-OH-Ket, we used a high performance liquid chromatography on an HSA-CSP. Under the chromatographic conditions used in the study the enantiomers of *p*-OH-Ket and Ket were resolved after independent injections of *rac*-Ket and *rac*-*p*-OH-Ket. The capacity factors (*k*s), enantioselectivity factors (αs), and stereochemical resolution factors (R_ss) determined for *p*-OH-Ket enantiomers and (S)- and (R)-Ket are presented in Table 1 and representative chromatograms are presented in Figure 2A and B.

When *rac*-Ket and *rac*-*p*-OH-Ket were injected together, the peaks overlapped and direct injection into a HSA-CSP could not be used with clinical samples (Fig. 2C). Optimal separation was achieved by on-line coupling of an achiral analytical column (Merck LiChrosphere 100 RP-18) to the HSA-CSP (Figure 2D). The *k*s, αs, and R_s of the enantiomers of Ket and its metabolite observed on the coupled system are presented (Table 1). Chromatographs obtained for blank and spiked human plasma and urine are shown in Figures 3A,B and 4A,B, respectively.

Linearity was observed in the calibration curves of Ket and *p*-OH-Ket enantiomers. The coefficients of correlation for all compounds in plasma and urine were between 0.991 and 0.999.

Intraday and interday reproducibilities of the method in plasma and urine are presented in Tables 2–5. The intra-assay variability for Ket and *p*-OH-Ket enantiomers was less than 6% in plasma and less than 8% in urine (Tables 2 and 4). The interday variability for *p*-OH-Ket$_1$, *p*-OH-Ket$_2$, (S)-Ket, and (R)-Ket, in plasma, gave mean coefficients of variation of 6.40, 7.80, 8.60, and 9.50%, respectively (Table 3). The mean CVs in urine were 4.90% (*p*-OH-Ket$_1$), 5.13% (*p*-OH-Ket$_2$), 4.79% [(S)-Ket], and 4.03% [(R)-Ket] (Table 5).

The mean accuracies were determined using blinded unknowns. In plasma the mean accuracies ranged from 106.9% (*p*-OH-Ket$_2$) to 85.4% [(R)-Ket]. The mean accuracies determined in urine ranged from 100.8% [(S)-Ket] to 93.9% (*p*-OH-Ket$_2$). The mean recoveries of *p*-OH-Ket$_1$, *p*-OH-Ket$_2$, (S)- and (R)-Ket obtained from two spiked plasma were 68.7,

TABLE 4. Intraday validation of ketorolac (Ket) and p-OH-Ket in urine

Concentration (ng/ml)	Peak area ratio (mean ± SD, n = 10)	Coefficient of variation (%)
p-OH-Ket$_1$		
250	0053 ± 0.002	3.22
2500	0.596 ± 0.013	2.24
7500	1.382 ± 0.023	1.65
p-OH-Ket$_2$		
250	0.052 ± 0.002	3.62
2500	0.594 ± 0.016	2.66
7500	1.379 ± 0.025	1.79
(S)-Ket		
500	0.103 ± 0.006	5.95
5000	1.115 ± 0.018	1.64
12500	2.216 ± 0.024	1.08
(R)-Ket		
500	0.102 ± 0.008	7.90
5000	1.102 ± 0.019	1.72
12500	2.155 ± 0.042	1.93

TABLE 5. Interday validation of ketorolac (Ket) and p-OH-Ket in urine

Concentration (ng/ml)	Peak area ratio (mean ± SD, n = 12)	Coefficient of variation (%)
p-OH-Ket$_1$		
250	0.055 ± 0.002	4.27
2500	0.604 ± 0.028	4.63
7500	1.493 ± 0.087	5.81
p-OH-Ket$_2$		
250	0.054 ± 0.003	4.96
2500	0.601 ± 0.031	5.09
7500	1.472 ± 0.078	5.31
(S)-Ket		
500	0.102 ± 0.005	4.90
5000	1.104 ± 0.039	3.53
12500	2.323 ± 0.085	3.66
(R)-Ket		
500	0.101 ± 0.007	6.86
5000	1.087 ± 0.044	4.05
12500	2.258 ± 0.078	3.46

70.5, 96.9, and 97.9%, respectively, and from two spiked urine were 88.13, 91.4, 104.58, and 102.88%, respectively.

This method was applied to quantitate the enantiomers of p-OH-Ket and Ket in plasma and urine from patients undergoing cataract surgery and dosed with *rac*-Ket. Representative chromatograms of plasma and urine samples are presented in Figures 3C and 4C, respectively. The plasma concentration of the more active (S)-enantiomer was significantly lower than the corresponding concentration of the (R)-Ket, as has been previously reported.[3,4] The mean ratio (n = 6) of (R)-Ket to (S)-Ket was 3.89 ± 0.93. The limit of detection was 25 ng/ml for each enantiomer of p-OH-Ket, and 50 ng/ml for each enantiomer of Ket. However, in plasma, the concentration of the metabolite enantiomers were lower than the limit of detection.

The enantiospecific urinary excretion of Ket and p-OH-Ket has not been previously reported. In this study, the urine samples contained Ket in a mean R/S ratio of 1.26 ± 0.09 (n = 7), the mean p-OH-Ket$_1$/p-OH-Ket$_2$ ratio was 1.77 ± 0.47 (n = 7). Both Ket and p-OH-Ket are glucuronized in the carboxyl moiety to form the ester glucuronide.[7] After deconjugation no significant change in the R/S Ket (1.14 ± 0.2, n = 7) and p-OH-Ket$_1$/p-OH-Ket$_2$ (1.61 ± 0.26, n = 7) ratios were observed. Thus, the glucuronidation does not appear to be enantioselective.

LITERATURE CITED

1. Rooks W.H., Tomolonis A.J., Maloney P.J., Wallach M.B., Schuler M.E. The analgesic and anti-inflammatory profile of (±)-5-benzoyl-1,2-dihydro-3H-pyrrolo[1,2a]pyrrole-1-carboxylic acid (RS-37619). Agents Actions 12(5):684–690, 1982.
2. Flach J.A., Jampol L.M., Weinberg D., Kraff M.C., Yannuzzi L.A., Campo R.V., Neumann A.C., Cupples H.P., Lefler H., Pulido J.S., Lavelle C. Improvement in visual acuity in chronic aphakic and pseudophakic cystoid macular edema after treatment with topical 0.5% ketorolac tromethamine. Am. J. Ophtholmol. 112:514–519, 1991.
3. Guzman A., Yuste F., Toscano R.A., Young J.M., Varn Horn A.R., Muchowski J.M. Absolute configuration of (−)-5-benzoyl-1,2-dihydro-3H-pyrrolo[1,2a]pyrrole-1-carboxylic acid. J. Med. Chem. 29:589–591, 1986.
4. Hayball P.J., Wrobel J., Tamblyn J.G., Nation R.L. The pharmacokinetics of ketorolac enantiomers following intramuscular administration of the racemate. Br. J. Clin. Pharmacol. 37:75–78, 1994.
5. Mroszczak E.J., Jung D., Yee J., Bynum L., Sevelius H., Massey I. Ketorolac tromethamine pharmacokinetics and metabolism after intravenous, intramuscular, and oral administration in humans and animals. Pharmacotherapy 10(6 Pt 2):33S–39S, 1990.
6. Jung D., Mroszczak E.J., Wu A., Ling, T.L., Sevelius H., Bynum L. Pharmacokinetics of ketorolac and p-hdyroxyketorolac following oral and intramuscular administration of ketorolac tromethamine. Pharm. Res. 6(1):62–65, 1989.
7. Mroszczak E.J., Lee F.W., Combs D., Sarnquist F.H., Huang B-L., Wu A., Tokes L.G., Maddox M.L., Cho D.K. Ketorolac tromethamine absorption, distribution, metabolism, excretion, and pharmacokinetics in animals and humans. Drug Metab. Dispos. 15(5):618–626, 1987.
8. Ling T.L., Combs D.L. Ocular bioavailability and tissue distribution of [^{14}C]ketorolac tromethamine in rabbits. J. Pharm. Sci. 76(4):289–294,1987.
9. Hayball P.J., Tambyn J.G., Holden Y., Wrobel J. Stereoselective analysis of ketorolac in human plasma by high-performance liquid chromatography. Chirality 4:1–5, 1992.
10. Jamali F., Mehvar R., Lemko C., Eradiri O. Application of a stereospecific high-performance liquid chromatography assay to a pharmacokinetic study of etodolac enantiomers in humans. J. Pharm. Sci. 77(11):963–966, 1988.