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An indirect (derivatization) and a direct HPLC method for the determination of the enantiomers of ketorolac in plasma

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Abstract

An indirect and a direct HPLC method for the quantification of the (*R*) and (*S*) enantiomers of ketorolac are described here. The indirect method employs the chiral amine (+)-*R*-1-(1-naphthyl)ethylamine to form disastereomeric amides; separation of the disastereomeric derivates is achieved by normal-phase HPLC with a mobile phase of ethyl acetate–hexane. The direct method uses a C18 solid-phase extraction column to extract ketorolac enantiomers from plasma; the reconstituted extract is then injected onto an α_1-acid glycoprotein chiral column using a mobile phase of isopropanol–phosphate buffer (0.05 M; pH 5.5). Both methods are reproducible, accurate, and stereospecific, and both have equivalent quantification limits (0.02 μg ml^{-1} of plasma for each enantiomer), ranges (0.02–2.0 μg per aliquot of plasma), precision (% relative standard deviations of $\leqslant$10.5% and $\leqslant$10.8% for (*R*)- and (*S*)-ketorolac respectively), and accuracy (mean recoveries of 88.4–110% and 90.1–110% for (*R*)- and (*S*)-ketorolac respectively). Results of analyses of clinical samples by the two methods showed excellent agreement (slope near 1.0 and coefficients of correlation between 0.9740 and 0.9864 for both enantiomers).

Keywords: Enantiomeric separations; Enantioselectivity; Ketorolac; Nonsteroidal antiinflammatory drugs

1. Introduction

Ketorolac tromethamine (**I**, Fig. 1) is a potent non-narcotic analgesic, with cyclooxygenase inhibitory activity [1–4]. It is approved for the treatment of mild to moderately severe post-operative pain and is marketed as a racemic mixture of (−) *S* and (+) *R* enantiomers. Animal studies have shown that the pharmacological activity of ketorolac resides in the (−) *S* enantiomer and that the (+) *R* enantiomer is pharmacologically inactive [5].

Pharmacokinetics of ketorolac as a total of the two enantiomers in animals and humans have been extensively reported [6–8]. To obtain stereospecific kinetics of each enantiomer and to investigate the potential interconversation of the

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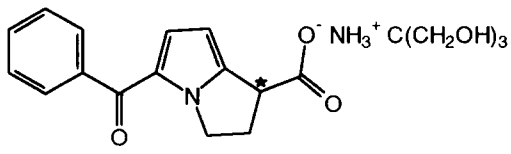
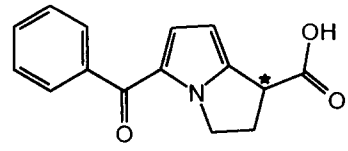
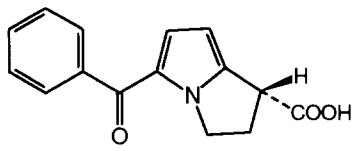


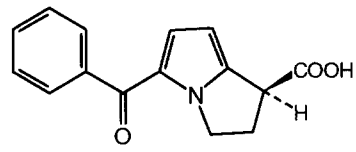
Racemic Ketorolac Tromethamine
Structure I



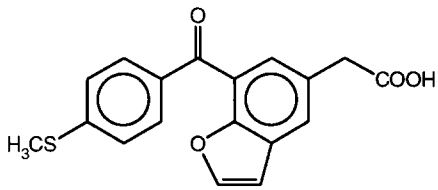
Racemic Ketorolac
Structure II



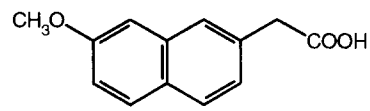
(*R*)-Ketorolac
Structure IIa



(*S*)-Ketorolac
Structure IIb



Internal Standard I
Structure III



Internal Standard II
Structure IV

*Chiral center

Fig. 1. Structure of racemic ketorolac tromethamine, racemic ketorolac, (*R*)-ketorolac, (*S*)-ketorolac, internal standard **I** (indirect method), and internal standard **II** (direct method).

two enantiomers in vivo, two HPLC methods—(1) derivatization to form diastereomers that were separated by achiral HPLC and (2) direct HPLC using a chiral-phase column—for the quantification of the ketorolac enantiomers in plasma were developed and are reported here, along with precision and accuracy data for both methods.

Other methods have recently been reported for the resolution of the ketorolac enantiomers either by first forming disastereomers and separating them on achiral columns [8,9] or by using chiral columns for the separation [10–14]. With the exception of one method [13], these methods have quantification limits two to five times higher than the methods reported here, and were not adequately sensitive for the study of the in vivo conversion of the enantiomers. One method [13] has comparable sensitivity to the method reported here but did not address stereospecificity and enantiomeric interconversion during the assay procedure because the authors had no reference samples of the individual enantiomers. For the

methods reported here, stability and stereospecificity issues have been addressed.

The indirect (derivatization) method described herein was developed during the early development phase of the drug, when reliable chiral columns were not yet available. The direct method, which uses and α_1-acid glycoprotein (AGP) column, was developed later. The description and validation of each method and an evaluation of the equivalency of the two methods are included in this report.

2. Experimental

2.1. Indirect method

2.1.1. Chemicals and supplies

Racemic ketorolac, (±)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid (**II**, Fig. 1); (*R*)-ketorolac, (+)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid (**IIa**, Fig. 1; reportedly 98.72% enantiomerically pure); (*S*)-ketorolac, (−)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid (**IIb**, Fig. 1; reportedly 98.02% enantiomerically pure); and internal standard **I**, RS-82917-000, 7-[4-methylthiobenzoyl]-benzofuran-5-yl acetic acid (**III**, Fig. 1) were supplied by Syntex Research (Palo Alto, CA). 1-Hydroxybenzotrizole, (+)-*R*-1-(1-naphthyl)ethylamine, enantiomeric purity ≥ 99%, and *N*,*N*′-dicylohexylcarbodiimide were purchased from Aldrich Chemical (Milwaukee, WI). Analytical-reagent-grade pyridine, sodium acetate, and glacial acetic acid were obtained from Mallinckrodt (St. Louis, MO); HPLC-grade acetonitrile, ethyl acetate, hexane, and dichloromethane were supplied by Burdick and Jackson Laboratories (Muskegon, MI). Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA). Heparinized human control plasma (blank plasma) was obtained from normal, healthy volunteers from the Clinical Studies Unit, Syntex Research (Palo Alto, CA). The following solutions were prepared in house: an aqueous solution of sodium acetate buffer (pH 3; 0.5 M); a solution of *N*,*N*′-dicyclohexylcarbodiimide in dichloromethane (1 mg ml^{-1}; a solution of 1-hydroxybenzotriazole in dichloromethane containing 1% pyridine (1 mg ml^{-1}); and a solution of (+)-*R*-1-(1-naphthyl)ethylamine in dichloromethane (1 mg ml^{-1}), which is prepared weekly and stored refrigerated.

2.1.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard Model 1090 L chromatograph with ternary solvent delivery system and an autosampler (Santa Clara, CA), a Kratos Spectroflow 773 variable wavelength UV detector (Kratos Analytical Instruments, Ramsey, NJ), and a Nelson 6000 Laboratory Data System (PE-Nelson, Cupertino, CA).

2.1.3. Chromatographic conditions

For the determination of (*R*)-ketorolac and (*S*)-ketorolac enantiomers, aliquots of sample extract (10 μl) were injected onto a Microsorb Silica, 5 μm, 150 mm × 4.6 mm column (Rainin Instrument Co., Emeryville, CA) maintained at room temperature using a mobile phase of ethyl acetate–hexane (40:60, v/v) and a flow rate of 1.0 ml min^{-1}. The UV detector was set at 317 nm.

2.1.4. Spiking procedure

A stock solution was prepared by dissolving racemic ketorolac in acetonitrile. The stock solution was further diluted with acetonitrile–water (9:1, v/v) to prepare spiking solutions at concentrations of 0.4, 1.0, 2.0, 5.0, 10 and 20 μg ml^{-1}. Stock solutions of (*R*)-ketorolac alone and (*S*)-ketorolac alone were prepared by dissolving (*R*)-ketorolac and (*S*)-ketorolac in acetonitrile. The stock solutions were further diluted with acetonitrile–water (9:1, v/v) to prepare spiking solutions for each enantiomer at a concentration of 10 μg ml^{-1}. A stock internal standard **I** solution was prepared by dissolving internal standard **I** in acetonitrile; it was further diluted with acetonitrile–water (9:1, v/v) to prepare an internal standard **I** spiking solution of 2.0 μg ml^{-1}.

Racemic ketorolac was spiked into blank plasma to prepare calibration standards used for the construction of calibration curves and for validation of the method. A set of calibration standards with nominal racemic ketorolac concen-

trations of 0.04, 0.10, 0.20, 0.50, 1.0, 2.0, and 4.0 μg ml^{-1} plasma (0.02, 0.05, 0.10, 0.25, 0.50, 1.0, and 2.0 μg of each enantiomer per milliliter) was prepared from the racemic ketorolac spiking solutions and aliquots of blank plasma by adding 0.1 ml of one of the racemic ketorolac spiking solutions (0.4, 1, 2, 5, 10, 20, or 40 μg ml^{-1}) to 1 ml of blank plasma.

2.1.5. Extraction of calibration standards

A calibration standard in a 15 ml test tube was spiked with 100 μl of the internal standard **I** spiking solution and acidified with 100 μl of sodium acetate buffer (pH 3; 0.5 M). The mixture was briefly vortexed, and 6 ml of ethyl acetate–hexane (30:70, v/v) was added. The resulting mixture was vortexed for 5 min, and the phases were allowed to settle for 5 min. The upper organic layer was transferred to a clean test tube and evaporated to dryness under a stream of nitrogen at 38°C.

2.1.6. Derivatization

The residue obtained was redissolved in 1 ml of dichloromethane, and added to it were 50 μl of the 1 mg ml^{-1} solution of 1-hydroxybenzoltriazole containing 1% pyridine, 100 μl of the 1 mg ml^{-1} solution of *N,N'*-dicyclohexylcarbodiimide, and 100 μl of the 1 mg ml^{-1} solution of (+)-*R*-1-(1-naphthyl)ethylamine. The tube was capped, vortexed for 1 min, and then allowed to incubate at room temperature for 2.5–24 h. The sample was then evaporated to dryness under a stream of nitrogen at 38°C. The residue obtained was reconsituted in 150 μl of ethyl acetate–hexane (40:60, v/v); the tube contents were vortexed and sonicated, and were then ready for HPLC analysis.

2.1.7. Extraction of clincial samples

Samples of heparinized plasma obtained from healthy volunteers or patients treated with ketorolac tromethamine were stored at −20°C before analysis. Samples were thawed at room temperature, vortexed briefly, and centrifuged for 2 min at approximately 2500 rev min^{-1}; an aliquot of 0.05–1.0 ml was dispensed for analysis. When less than 1.0 ml was used for analysis, blank plama was added to bring the total volume to 1.0 ml. Samples were extracted and subjected to derivatization using the same procedure described for the calibration standards. The concentrations of the ketorolac enantiomers were calculated by reference to calibration curves generated from calibration standards analyzed together with each batch of samples.

2.1.8. Data handling and calculations

Linear least-squares regression was performed on the peak height ratio [amide of (*R*)-or (*S*)-ketorolac:amide of the internal standard] versus the concentration of each enantiomer in the calibration standard to construct a linear standard curve of the form peak height ratio = a (concentration) + b. Calibration standards from 0.02–0.50 μg ml^{-1} for each enantiomer were used in the unweighted linear regression to construct a calibration curve, and calibration standards of higher concentrations were used to verify extrapolations of the curve up to 2.0 μg ml^{-1} for each enantiomer of ketorolac. The slope and intercept of the calibration curve for (*R*)- or (*S*)-ketorolac were used for the calculation of the concentrations of the respective enaniomers in unknown samples, with appropriate corrections for sample aliquot volumes that were less than 1.0 ml.

2.1.9. Preparation of quality control samples

Quality control samples (QCs) prepared by spiking racemic ketorolac into blank plasma were stored at −20°C in a manner similar to that used for the clinical samples. QCs were prepared at the following three concentrations: low QC (0.050 μg ml^{-1} for each enantiomer); mid QC (0.498 μg ml^{-1} for each enantiomer); and high QC (1.83 μg ml^{-1} for each enantiomer). To prepare the bulk QCs, racemic ketorolac was dissolved in acetonitrile and diluted with acetonitrile–water (9:1, v/v) to prepare a spiking solution. To blank plasma was added an appropriate amount of spiking solution; the mixture was then swirled briefly and stirred with a magnetic stirrer for 10 min before being apportioned into polypropylene tubes for storage at −20°C. Two QCs of each of the three different concentrations were analyzed with each batch of clinical samples to monitor the performance of the method for routine use.

Table 1
Precision and accuracy for (*R*) and (*S*) enantiomers of ketorolac in spiked blank plasma samples determined by the indirect method

Enantiomer	Nominal conc. (μg ml^{-1})	Intra-assay analysis ($n = 4$)			Inter-assay analysis ($n = 4$)		
		Mean conc. found (μg ml^{-1})	%RSD	% Recovery[a]	Mean conc. found (μg ml^{-1})	%RSD	% Recovery[a]
(*R*)-Ketorolac	0.020	0.022	9.1	110	0.021	0	105
	0.050	0.051	3.9	102	0.050	6.0	100
	0.100	0.100	3.0	100	0.096	3.1	96.0
	0.250	0.270	1.5	108	0.254	0.4	102
	0.500	0.517	2.3	103	0.499	0.2	99.8
	1.00	1.06	2.0	106	0.994	2.3	99.4
	2.00	2.13	1.4	107	2.01	3.4	101
(*S*)-Ketorolac	0.020	0.020	3.0	100	0.021	3.8	105
	0.050	0.049	2.0	98	0.050	4.0	100
	0.100	0.100	3.0	100	0.097	2.1	97.0
	0.250	0.268	1.9	107	0.252	0.8	101
	0.500	0.506	1.6	101	0.499	0.2	99.8
	1.00	1.04	1.6	104	0.994	3.7	99.4
	2.00	2.07	1.5	104	2.01	2.7	101

[a] % Recovery is defined as found concentration/nominal concentration, expressed as a percentage.

2.2. Direct method

2.2.1. Chemicals and supplies

Racemic ketorolac tromethamine, (±)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid, tris(hydroxymethyl)aminomethane salt (**I**, Fig. 1); racemic ketorolac, (±)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid (**II**, Fig. 1); (*R*)-ketorolac, (+)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid (**IIa**, Fig. 1; reportedly 100.0% enantiomerically pure, different lot from that used for indirect method); (*S*) -ketorolac, (−)-5-benzoyl-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid (**IIb**, Fig. 1; reportedly 99.7% enantiomerically pure, different from that used for indirect method); and internal standard **II**, RS-3418, 6-methoxy-2-naphthylacetic acid (**IV**, Fig. 1) were supplied by Syntex Research (Pala Alto, CA). HPLC-grade methanol, acetonitrile, isopropanol, hexane, and ethyl acetate were purchased from Burdick and Jackson Laboratories (Muskegon, MI), and water was purified by a Miller-Q water purification system (Millipore, Bedford, MA). Reagent-grade phosphoric acid, 85%, analytical-grade sodium acetate, glacial acetic acid, potassium phosphate monobasic, and sodium hydroxide were purchased from Mallinckrodt (St. Louis, MO and Paris, KY). Sodium azide was purchased from J.T. Baker (Phillipsburg, NJ). Heparinized human control plasma (blank plasma) was obtained from normal, healthy volunteers from the Clinical Studies Unit, Syntex Research (Palo Alto, CA). Solid-phase extraction columns, C18 (100 mg of sorbent mass, 6 ml volume, custom-packed), were purchased from Varian Associates (Harbor City, CA). Aqueous solutions of sodium acetate buffer (pH 3; 0.02 M) and phosphate buffer (pH 5.5; 0.05 M, prepared by adjusting the pH of a 0.05 M potassium phosphate monobasic solution with a 0.1 N sodium hydroxide solution) were prepared in house.

2.2.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard Model 1090 L chromatograph with ternary solvent delivery system and an autosampler (Santa Clara, CA), a Kratos Spectroflow 783A variable wavelength UV detector (Kratos Analytical Instruments, Ramsey, NJ), and a Nelson 6000

Table 2
Precision and accuracy of QC samples determined by the indirect method

Parameter	Low QC (*R*) and (*S*) 0.050 μg ml^{-1}		Mid QC (*R*) and (*S*) 0.498 μg ml^{-1}		High QC (*R*) and (*S*) 1.83 μg ml^{-1}	
	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)
Intra-assay						
Mean (μg ml^{-1})	0.0463	0.0463	0.504	0.509	1.85	1.84
%RSD	2.07	2.07	2.67	2.85	2.56	3.04
% Recovery[a]	92.6	92.6	101	102	101	101
n	4	4	4	4	4	4
Inter-assay						
Mean (μg ml^{-1})	0.0442	0.0461	0.491	0.497	1.82	1.84
%RSD	10.5	10.8	6.74	6.74	7.97	7.88
% Recovery[a]	88.4	92.2	98.6	99.8	99.5	101
n	20	20	20	20	20	20

[a] % Recovery is defined as found concentration/nominal concentration, expressed as a percentage.

Laboratory Data System (PE-Nelson, Cupertino, CA).

2.2.3. Chromatographic conditions

For the determination of (*R*)-ketorolac and (*S*)-ketorolac enantiomers, aliquots of sample extract (15 μl) were injected onto a Chiral AGP, 5 μm, 100 mm × 4.0 mm column (Chrom Tech, Apple Valley, MN) maintained at room temperature using a mobile phase of isopropanol–phospate buffer (pH 5.5; 0.05 M, containing 2.5 mM sodium azide as preservative) at a ratio of 5:95 (v/v) and a flow rate of 0.4 ml min^{-1}. The UV detector was set at 317 nm. A Chiral AGP guard column (Chrom Tech, Apple Valley, MN) was connected to the analytical column.

2.2.4. Spiking procedure

A stock solution was prepared by dissolving racemic ketorolac tromethamine in acetonitrile. The stock solution was further diluted with acetonitrile–water (9:1, v/v) to prepare spiking solutions at equivalent ketorolac concentrations of 0.4, 1, 2, 5, 10, 20, and 40 μg ml^{-1}. A stock internal standard **II** solution was prepared by dissolving the internal standard **II** in methanol–water (9:1, v/v); it was further diluted with metnanol–water (9:1, v/v) to prepare an internal standard **II** spiking solution of 25 μg ml^{-1}. Racemic ketorlac was spiked into blank plasma to prepare calibration standards used for the construction of calibration curves and for validation of the method. To 1 ml aliquots of blank plasma was added 0.1 ml of racemic ketorolac spiking solution (0.4, 1, 2, 5, 10, 20, or 40 μg ml^{-1}) to give calibration standard with nominal racemic ketorlac concentrations of 0.04, 0.10, 0.20, 0.50, 1.0, 2.0, and 4.0 μg ml^{-1} plasma (0.02, 0.05, 0.10, 0.25, 0.50, 1.0 and 2.0 μg of each enantiomer per milliliter).

2.2.5. Extraction of calibration standards

The C18 solid-phase extraction columns were used with gravity flow for both preconditioning and sample extraction. Columns were preconditioned first with 1 ml of methanol and then with 1 ml of water and allowed to drain thoroughly. Columns were used within 1 h of preconditioning. To a test tube containing a calibration standard were added 0.1 ml of internal standard **II** spiking solution and 3.0 ml of the sodium acetate buffer (pH 3.0; 0.02 M). The mixture was vortexed briefly and then applied to a preconditioned C18 solid-phase extraction column. The solution was allowed to pass through the column. 1 ml of water was added to wash the test tube that had

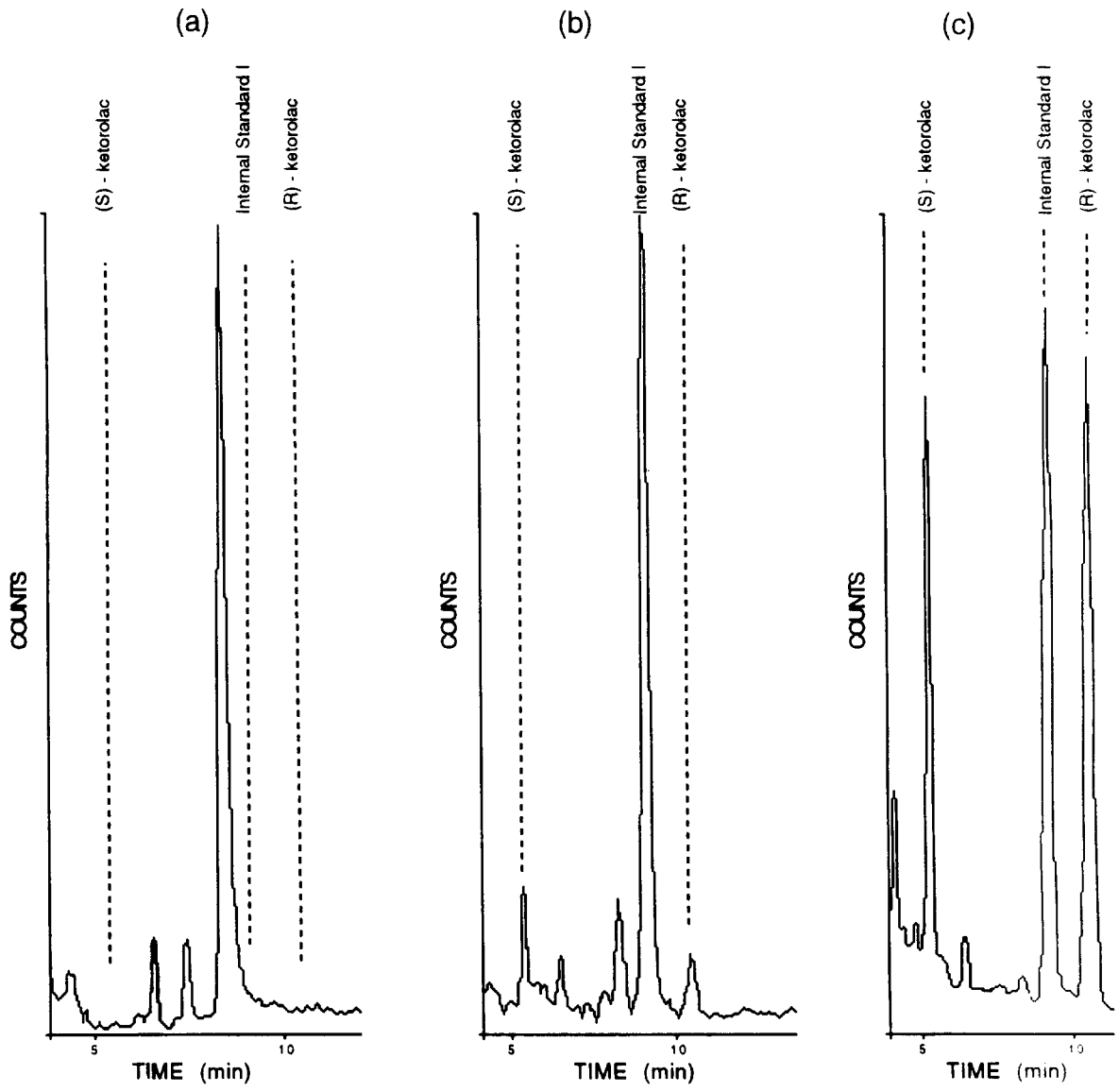


Fig. 2. Indirect method: chromatograms of (a) blank plasma from an untreated subject; (b) blank plasma spiked with 0.040 μg of racemic ketorolac per milliliter; (c) a patient plasma sample 2 h after oral administration of 10 mg of racemic ketorolac tromethamine.

contained the solution and vortexed. This wash water was then applied to the same solid-phase extraction column to which solution had been applied. The solid-phase column was then washed with 1 ml of methanol–water (2:8, v/v) and next with 2 ml of hexane (for the hexane wash, it was occasionally necessary to begin the flow with a pulse of air or nitrogen). Analytes were then eluted into a test tube with 2 ml of ethyl acetate–hexane (3:7, v/v). The eluate was evaporated to dryness at 38°C under a stream of nitrogen. The sample was reconsituted in the tube with 0.15 ml of the HPLC mobile phase, isopropanol–phosphate buffer (pH 5.5; 0.05 M; containing 2.5 mM sodium azide) (5:95, v/v). Tubes were sonicated for 30 s and vortexed for 30 s. Tube contents were then transferred to HPLC vials.

Table 3
Results of the stereospecificity study of the indirect method

Enantiomer	n	Concentration added ($\mu g\ ml^{-1}$)	(*S*) Mean Conc. found ($\mu g\ ml^{-1}$)	(*S*)RSD%	(*R*) Mean Con. found ($\mu g\ ml^{-1}$)	(*R*)RSD%	Mean Conbined Conc. (*S*) and (*R*) ($\mu g\ ml^{-1}$)	% (*S*)	% (*R*)
(*S*)-Ketorolac	21	1.00	0.973	3.31	0.051	10.7	1.024	95.0	5.0
(*R*)-Ketorolac	21	1.00	0.028	14.7	0.955	3.33	0.983	2.8	97.2

2.2.6. Extraction of clinical samples

Samples of heparinized plasma obtained from healthy volunteers or patients treated with ketorolac tromethamine were stored at −20°C before analysis. Samples were thawed at room temperature, vortexed briefly, and centrifuged for 2 min at approximately 2500 rev min^{-1}; an aliquot of 0.2–1.0 ml was dispensed for analysis. When less than 1.0 ml was used for analysis, blank plasma was added to bring the total volume to 1.0 ml. Samples were extracted using the same procedure described for the calibration standards. The concentrations of (*R*)- and (*S*)-ketorolac were calculated by reference to calibration curves generated from calibration standards analyzed together with each batch of samples.

2.2.7. Data handling and calculations

Linear least-squares regression was performed on the peak height ratio [(*R*)- or (*S*)-ketorolac:internal standard] versus the concentration of each enantiomer in the calibration standard to construct a linear standard curve of the form peak height ratio $= a$ (concentration) $+ b$. Calibration standards from 0.020–0.25 $\mu g\ ml^{-1}$ for (*R*)- or (*S*)-ketorolac were used in the unweighted linear regression to construct a calibration curve, and calibration standards of higher concentrations were used to verify extrapolations of the curve up to 2.0 $\mu g\ ml^{-1}$ for (*R*)- or (*S*)-ketorolac. The slope and intercept for the (*R*)- or (*S*)-ketorolac calibration curve were used for the calculation of the concentrations of the respective enantiomers in unknown samples, with appropriate corrections for sample aliquot volumes that were less than 1.0 ml.

2.2.8. Preparation of QCs

QCs prepared by spiking (*R*)-ketorolac and (*S*)-ketorolac into blank plasma were stored at −20°C in a manner similar to that used for the clinical samples. QCs were prepared at the following five concentrations: (a) low QC (0.0398 μg of (*R*)-ketorolac and 0.0398 μg of (*S*)-ketorolac ml^{-1}); (b) mid QC (0.397 μg of (*R*)-ketorolac and 0.397 μg of (*S*)-ketorolac ml^{-1}); (c) high QC (3.52 μg of (*R*)-ketorolac and 3.52 μg of (*S*)-ketorolac ml^{-1}); (d) low/high QC (0.0395 μg of (*R*)-ketorolac and 0.889 μg of (*S*)-ketorolac ml^{-1}); (e) high/low QC (0.889 μg of (*R*)-ketorolac and 0.0395 μg of (*S*)-ketorolac ml^{-1}). To prepare the bulk QCs, (*R*)-ketorolac and (*S*)-ketorolac were dissolved separately in acetonitrile. Each solution was then further diluted with acetonitrile–water (9:1, v/v) to prepare a spiking solution. To blank plasma was added an appropriate amount of a spiking solution of the (*R*)- on (*S*-)-ketorolac; the mixture was then swirled briefly and stirred with a magnetic stirrer for 10 min before being apportioned into polypropylene tubes for storage at −20°C. Two QCs of each of three different concentrations (either low, mid, and high, or low/high, mid, and high/low) were analyzed with each batch of clinical samples to monitor the performance of the method during routine use.

3. Results and discussion

3.1. Indirect method

3.1.1. Method development and optimization

The derivatization of the (*R*) and (*S*) enantiomers of ketorolac with (+)-*R*-1-(1-naphthyl)-ethylamine was first reported by Guzman et al.

Table 4
Precision and accuracy for (*R*) and (*S*) enantiomers of ketorolac in spiked blank plasma samples determined by the direct method

Enantiomer	Nominal conc. (μg ml^{-1})	Intra-assay analysis ($n = 4$)			Inter-assay analysis ($n = 4$)		
		Mean conc. found (μg ml^{-1})	%RSD	% Recovery[a]	Mean conc. found (μg ml^{-1})	%RSD	% Recovery[a]
(*R*)-Ketorolac	0.020	0.0185	3.31	92.5	0.0214	6.07	107
	0.050	0.0481	3.97	96.2	0.0487	3.78	97.4
	0.100	0.101	2.22	101	0.0969	6.83	96.9
	0.250	0.248	2.89	99.2	0.242	5.25	96.8
	1.00	1.01	1.40	101	1.02	6.27	102
	2.00	2.03	2.14	102	1.93	4.19	96.5
(*S*)-Ketorolac	0.020	0.0191	2.16	95.5	0.0219	6.85	110
	0.050	0.0486	3.93	97.2	0.0484	6.26	96.8
	0.100	0.100	2.33	100	0.0985	4.37	98.5
	0.250	0.250	1.51	100	0.245	5.18	98.0
	1.00	1.01	1.43	101	1.00	3.65	100
	2.00	2.02	2.39	101	1.88	1.81	94.0

[a] % Recovery is defined as found concentration/nominal concentration, expressed as a percentage.

[5], who separated the diastereomeric amides by flash chromatography on silica gel. In the method reported here, the diastereomeric amides were formed following reaction of racemic ketorolac with (+)-*R*-1-(naphthyl)-ethylamine in the presence of 1-hydroxybenzotriazole and *N,N'*-dicyclohexylcarbodiimide and separated by normal-phase HPLC. A series of experiments that examined the effect of the reaction time and the concentration of each reagent on the yield of the amides formed revealed optinum conditions of 50 μg of 1-hydroxybenzotriazole and 100 μg each of (+)-*R*-1-(naphthyl)-ethylamine and *N,N'*-dicyclohexylcarbodiimide per sample at room temperature with a reaction time of 2.5 h or longer. Equivalent results were obtained for incubation times of 2.5–24 h; 2.5 h was generally the most convenient time for this work. Essentially equal amounts of the diastereomeric amides of ketorolac are formed during derivatization.

3.1.2. Quantification limit and linearity

The quantification limit of the assay for each enantiomer is 0.020 μg ml^{-1} plasma using the maximum volume of 1 ml for analysis. Concentrations below 0.02 μg ml^{-1} are reported as being below the quantification limit of the method (BQL). The lower limit of quantification (LLOQ) was set at the lowest concentration that gave a signal-to-noise ratio (S/N) of ≥ 6 for each enantiomer. At the LLOQ (0.02 μg ml^{-1}), the S/N of HPLC peaks for the amides of (*R*)- and (*S*)-ketorolac are approximately 6:1 and 10:1 respectively. The linear range of the method, using 0.05–1.0 ml of plasma for analysis, is 0.02–2.0 μg per aliquot of plasma.

3.1.3. Precision and accuracy

The precision of the assay was assessed by intra-assay (within day) and inter-assay (between day) relative standard deviations (% RSDs) of the method (Table 1). The accuracy of the method was evaluated by the recovery, defined as the ratio of the concentration of ketorolac enantiomers found in the sample to that added to the sample (found/added). Data obtained from the analysis of individually spiked blank plasma samples, prepared in a manner identical to that used for preparation of calibration standards, are presented in Table 1. Corresponding data from the analysis of QC samples are presented in Table 2. All RSDs for both spiked calibration standards and QC samples were less than 11%. Recoveries were in the range 88.4–110%.

Table 5
Precision and accuracy of QC samples determined by the direct method

Parameter	Low QC (*R*) and (*S*) 0.0398 μg ml^{-1}		Mid QC (*R*) and (*S*) 0.0397 μg ml^{-1}		High QC (*R*) and (*S*) 3.52 μg ml^{-1}		High/low QC (*R*) 0.889 μg ml^{-1} (*S*) 0.0395 μg ml^{-1}		Low/high QC (*R*) 0.0395 μg ml^{-1} (*S*) 0.889 μg ml^{-1}	
	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)	(*R*)	(*S*)
Intra-assay										
Mean (μg ml^{-1})	0.0380	0.0379	0.387	0.372	3.45	3.23	0.840	0.0473	0.0435	0.852
%RSD	1.22	1.40	1.14	1.43	1.79	1.52	2.52	2.88	3.13	2.16
% Recovery[a]	95.5	95.2	97.5	93.7	98.0	91.8	94.5	120	110	95.8
n	4	4	4	4	4	4	4	4	4	4
Inter-assay										
Mean (μg ml^{-1})	0.0359	0.0366	0.380	0.369	3.49	3.20	0.822	0.0470	0.0454	0.801
%RSD	5.13	5.71	4.11	5.04	5.39	6.06	4.31	5.70	7.49	3.93
% Recovery[a]	90.2	92.0	95.7	92.9	99.1	90.9	92.5	119	115	90.1
n	28	28	28	28	28	28	20	20	20	20

[a] % Recovery is defined as found concentration/nominal concentration, expressed as a percentage.

3.1.4. Specificity

Typical chromatograms obtained from human plasma extracts after derivatization are given in Fig. 2. Derivatives of both enantiomers were well resolved from internal standard **I** and from each other. No interference from endogenous compounds was observed in blank plasma from six individuals.

This method has also been applied to the determination of ketorolac enantiomers in rat and mouse plasma. The same procedures for sample preparation and HPLC were applied except that a longer column (Microsorb Silica, 5 μm,250 mm × 2.6 mm, Rainin Instrument Co., Emeryville, CA) was used to separate an interfering component from the analytes. Using this modification, no interference from endogenous compounds was observed in blank plasma.

3.1.5. Stereospecificity

To investigate the stereospecificity of the assay and the enantiomeric stability of samples, plasma samples spiked with 1.0 μg of either (*R*)- or (*S*)-ketorolac were analyzed repeatedly. The enantiomeric purities of the (*R*)- and (*S*)-ketorolac used were reported to be 98.72% and 98.02% respectively. The derivatization reagent, (+)-*R*-1-(naphthyl)-ethylamine had ⩾99% enantiomeric purity by label claim. The data indicate that the method is specific for each enantiomer and that less than 3% of one enantiomer is measured as the other enantiomer (Table 3).

During the routine use of this method, the derivatization step was monitored by the analysis of two samples of plasma, one spiked with 1 μg of (*R*)-ketorolac alone and the other spiked with 1 μg of (*S*)-ketorolac alone, with each set of unknowns, to ensure that the derivatization reagent, (+)-*R*-1-(1-naphthyl)-ethylamine, had retained its enantiomeric purity.

3.1.6. Stability

For discussion of the stability of racemic ketorolac and its enantiomers in plasma, see Section 3.2.6.

3.2. Direct method

3.2.1. Method development and optimization

Because of concerns over the stability of the protein column, the effects of isopropanol concentration, buffer strength, pH, and flow rate on retention times and resolution were examined to determine the optimal mobile-phase conditions. They were determined to be isopropanol concentrations of less than 10%, phosphate concentra-

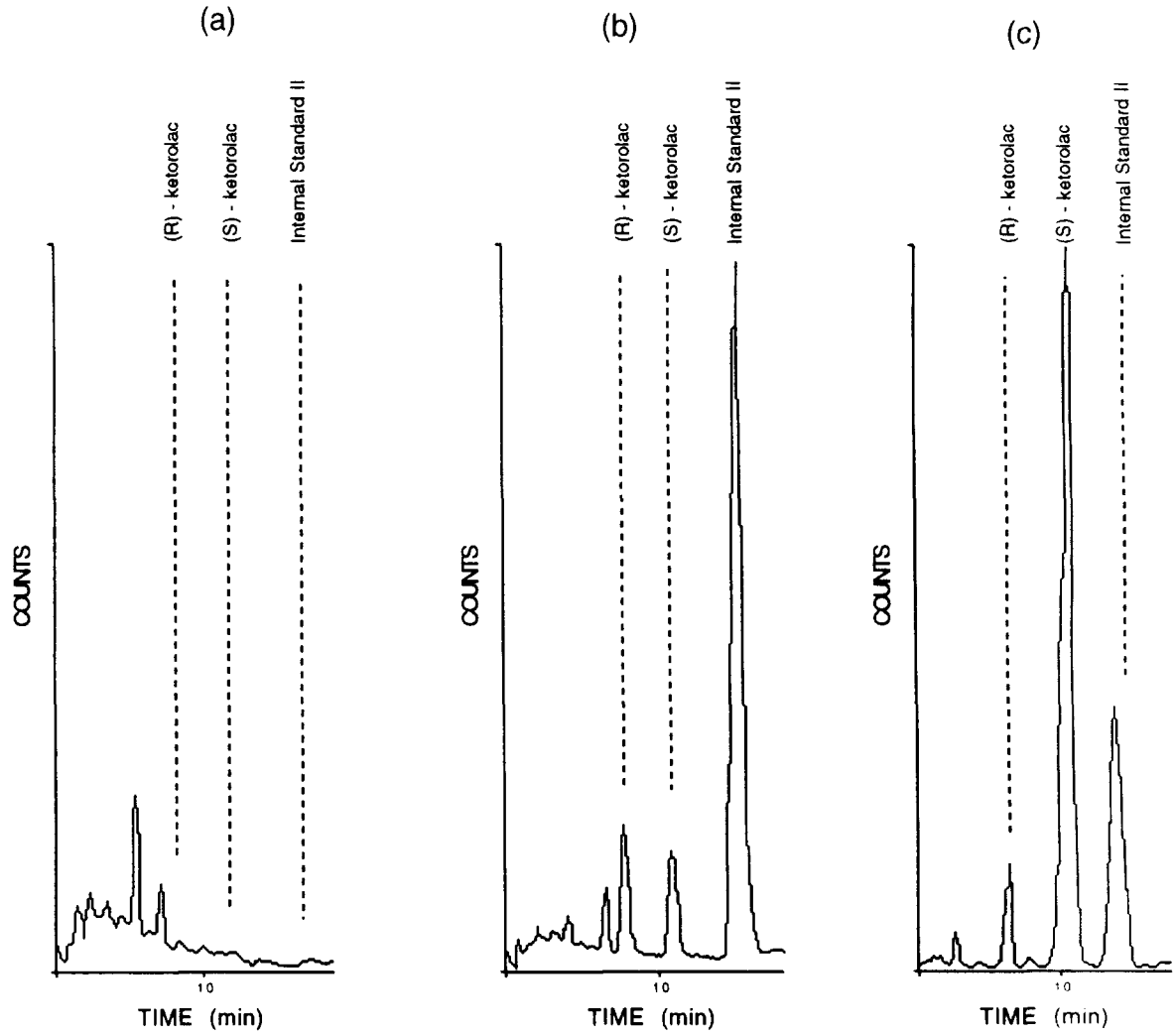


Fig. 3. Direct method: chromatograms of (a) blank plasma from an untreated subject; (b) blank plasma spiked with 0.040 μg of racemic ketorolac per milliliter; (c) a patient plasma sample 1 h after oral administration of 30 mg of racemic ketorolac tromethamine.

tions of 0.05 M or less, pH values of less than 6.0, and flow rates of less than 0.9 ml min^{-1}.

3.2.2. *Quantification limit and linearity*

The quantification limit of the method is 0.02 μg of (*R*)- or (*S*)-ketorolac per ml using a maximum volume of 1 ml of plasma for analysis. Concentrations below 0.02 μg ml^{-1} are reported as being BQL. The LLOQ was set at the same concentration as that for the indirect method, which had been developed and validated previously, provided that the S/N was ≥6. This allowed direct comparison of data collected by the two methods. At the LLOQ, the S/Ns of the HPLC peaks for (*R*)- and (*S*)-ketorolac are approximately 23:1 and 19:1 respectively. Although a LLOQ for each enantiomer of 0.02 μg ml^{-1} was adequate for the pharmacokinetic studies, potentially lower concentrations, should they be needed, could probably be achieved by this direct method. The linear range of the method is 0.02–2.00 μg of (*R*)- and (*S*)-ketorolac, and the con-

Table 6
Results of the stereospecificity study of the direct method

Enantiomer	n	Concentration added (μg ml^{-1})	(S) Mean conc. found (μg ml^{-1})	(R) Mean conc. found (μg ml^{-1})	Mean combined conc. (S) and (R) (μg ml^{-1})	%(S)	%(R)
(S)-Ketorolac	2	5.00	4.78	0.0645	4.85	98.6	1.33
(R)-Ketorolac	2	5.00	0.0896	4.92	5.01	1.79	98.2

centration range is 0.02–10.0 μg of each enantiomer per milliliter using 0.2–1.0 ml of plasma for analysis.

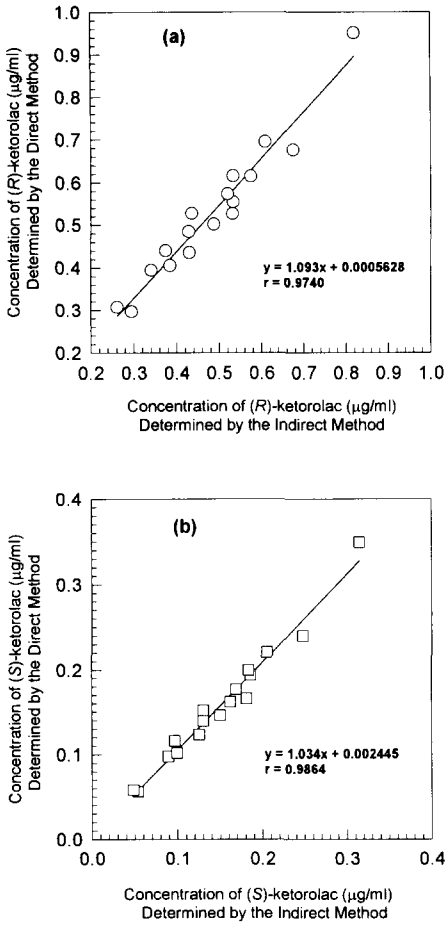


Fig. 4. Comparison of data obtained for the determination of (a) (R)-ketorolac and (b) (S)-ketorolac using the direct and indirect HPLC methods.

3.2.3. Precision and accuracy

The precision of the assay was assessed by the intra-assay (within day) and inter-assay (between day) % RSDs of the method. Data obtained from the analysis of individual samples spiked with ketorolac (Table 4) and from QC samples (Table 5) demonstrate % RSDs of less than 7.5% for all samples analyzed.

The accuracy of the method was evaluated by the recovery, defined as the ratio of the concentration of ketorolac enantiomer found by the assay to the known concentration added (found/added). Data obtained from the analysis of individually spiked blank plasma samples, prepared in a manner identical to that used for preparation of calibration standards (Table 4), and from QC

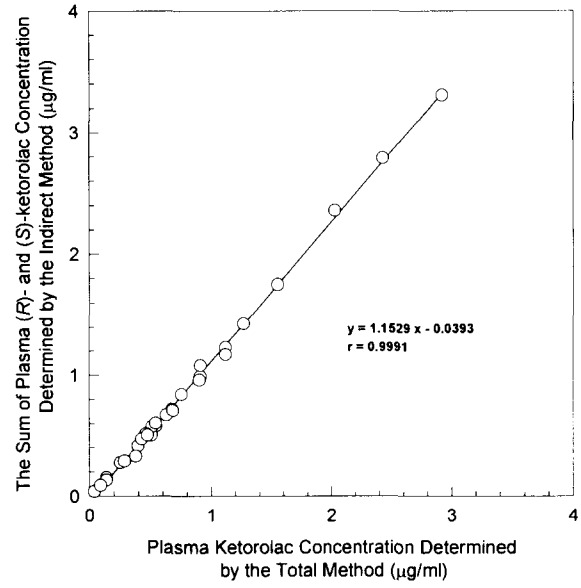


Fig. 5. Comparison of data obtained for the determination of the sum of (R)- and (S)- ketorolac using the indirect and the total ketorolac HPLC methods.

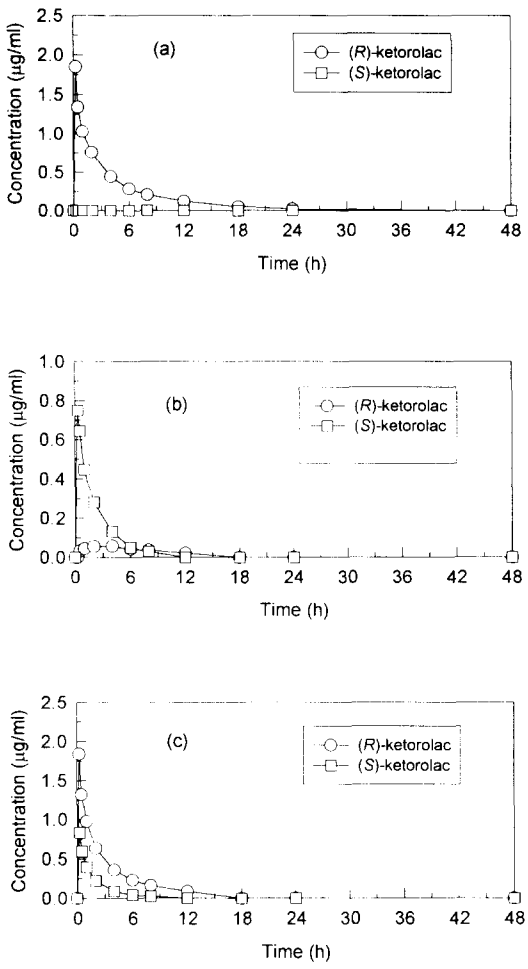


Fig. 6. Representative profiles from a healthy subject following oral administration of (a) 15 mg of (*R*)-ketorolac tromethamine, (b) 15 mg of (*S*)-ketorolac tromethamine, and (c) 30 mg of racemic ketorolac tromethamine. Concentrations of (*R*)- and (*S*)-ketorolac in plasma were determined using the direct method (values reported as BQL are plotted as 0 μg ml^{-1}).

samples (Table 5) demonstrated recoveries in the range 90.2%–110% except for the two QCs containing different concentrations of the enantiomers (low/high, high/low). The recoveries of these QC samples ranged from 92.5%–115% and from 90.1%–119% for (*R*)- and (*S*)-ketorolac respectively. Corrections in these recoveries were not made for small amounts of enantiomeric impurities present in the (*R*)- and (*S*)-ketorolac standards used to prepare the QC pools, and these small enantiomeric impurities (approximately $\leq$0.3%) contributed to the high recoveries noted for the lower concentration enantiomer in these samples.

3.2.4. *Specificity*

The analysis of blank human plasma obtained from six different sources showed no interfering peaks from endogenous substances at the retention times of (*R*)- and (*S*)-ketorolac and the internal standard. Chromatograms from blank plasma, from blank plasma spiked with 0.04 μg of racemic ketorolac per milliliter of plasma (0.02 μg ml^{-1} for each enantiomer), and from a plasma sample from a subject dosed with racemic ketorolac are presented in Fig. 3.

3.2.5. *Sterospecificity*

To investigate the stereospecificity of the method, plasma samples spiked with 5.0 μg of either (*R*)- or (*S*)-ketorolac per milliliter were analyzed in duplicate on the same day. The enantiomeric purities of the (*R*)- and (*S*)-ketorolac used were reported to be 100.0% and 99.7% respectively. The data indicate that the method is specific for each enantiomer and that less than 1.8% of one enantiomer is measured as the other enantiomer (Table 6).

3.2.6. *Stability*

QC samples containing ketorolac enantiomers at three different concentrations were analyzed within 4 days of preparation and again at various times after storage at −20°C; clinical samples containing ketorolac enantiomers at therapeutic concentrations were analyzed 7 weeks after collection and subsequent storage at −20°C and again after 18 months of storage at −20°C. The analytes were defined as stable if concentrations did not deviate from the initially determined concentrations by more than the accuracy parameters established during validation (within ± 10% of nominal) for QC samples spiked with equal amounts of the two enantiomers (see Table 5). By these criteria, enantiomer concentrations in these samples were stable for as long as 18 months (data not shown). Extracts prepared for HPLC analysis from calibration standards, QC samples,

and clinical study samples were analyzed by HPLC on the day samples were processed and after storage. They were stored refrigerated at 1–4°C or at room temperature (20–23°C) under normal laboratory conditions. By the stability criteria stated above, the resulting data indicate that ketorolac enantiomers in the extract are stable for at least 1 month when stored refrigerated and for 1 week when stored at room temperature (data not shown). Based on this information, the extract can await analysis on an HPLC system for up to 1 week, and can be analyzed or reanalyzed on the HPLC system withing 1 month of preparation when stored refrigerated.

3.2.7. Care of AGP column

The AGP column was stored in a solution of isopropanol–water (2:8, v/v) at room temperature or refrigerated at 1–4°C when not in use. To maintain column performance, the column was washed after each use with water for 30 min at a flow rate of 0.4 ml min^{-1} followed by isopropanol–water (2:8, v/v) for 30 min at a flow rate of 0.2 ml min^{-1}. To help restore deteriorating column performance, the orientation of the column was reversed and the column was washed with isopropanol–water (25:75, v/v) for 8 h at a flow rate of 0.2 ml min^{-1}. Mobile phase with greater than 25% isopropanol may cause protein precipitation and is not recommended. With routine use and proper maintenance, column performance was typically acceptable for 600–700 injections of plasma extracts.

3.3. Comparison of results of indirect, direct, and total racemic ketorolac HPLC methods

The direct method has quantification limits for (*R*)- and (*S*)-ketorolac identical to those reported for the indirect method. 17 samples of plasma collected during a clinical study were analyzed for (*R*)- and (*S*)-ketorolac using both methods. The two sets of data were compared by linear regression analysis (Fig. 4). The analysis indicates excellant agreement between the results obtained by the two methods. In another study, the results of the indirect method, which determines the concentrations of individual ketorolac enantiomers in plasma, were compared with the results of an HPLC method [15] that does not discriminate between the ketorolac enantiomers but determines total ketorolac concentration in plasma. For purposes of this study, the sum of the (*R*)- and (*S*)-ketorolac concentrations determined by the indirect method was compared to the total ketorolac concentration determined by the total ketorolac method. The two sets of data were compared by linear regression analysis (Fig. 5). The regression analysis indicates good agreement between the results obtained by the two methods.

3.4. Applications

Both methods have been applied to the analysis of plasma from patients treated with racemic ketorolac tromethamine and from healthy subjects treated either with racemic ketorolac tromethamine or with individual enantiomers of the drug. The studies were conducted to investigate the kinetics of each enantiomer and the in vivo interconversion of the enantiomers in humans. Representative profiles from a healthy subject following oral administration of racemic ketorolac tromethamine or individual enantiomers are presented in Fig. 6. In addition, the indirect method has been applied to the analysis of plasma from animals treated with either racemic ketorolac tromethamine or individual enantiomers of ketorolac tromethamine. Other applications of these methods have been presented in two abstracts [16,17] that described the pharmacokinetics of ketorolac enantiomers in humans and in animals.

4. Conclusions

Both the indirect (derivatization) and direct HPLC methods described here for the determination of the (*R*) and (*S*) enantiomer of ketorolac in human plasma are precise and accurate and can be used on clincial samples from patients who have been administered ketorolac tromethamine either intramuscularly, intravenously, or orally. Although the direct method is simpler and slightly faster, the column used is more expensive and has

a shorter lifetime than the column used for the indirect method. The two methods give equivalent analytical results.

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