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Research Article

A Validated High-Performance Liquid Chromatography-Tandem Mass Spectrometric (Lc-Ms/Ms) Method for Simultaneous Determination of R(+)-Ketorolac and S(−)-Ketorolac in Human Plasma and Its Application to a Bioequivalence Study

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We report a selective, accurate, and reproducible liquid chromatography-tandem mass spectrometric (LC-MS/MS) method that employs solid phase extraction for quantification of ketorolac enantiomers in human plasma. Resolution of R(+)-ketorolac and S(−)-ketorolac was achieved using a Chiral-AGP column and a mobile phase of ammonium formate buffer (10 mM, pH 4.70 ± 0.05):acetonitrile (85 : 15, v/v and 70 : 30, v/v) in a gradient time program. S(+)-etodolac was used as the internal standard (IS). Quantification was achieved using a positive electrospray ionization (ESI+) interface under multiple reaction monitoring (MRM) condition. The method was validated over the concentration range of 9.36–1198.69 ng/ml for R(+)-ketorolac and 6.07–776.74 ng/ml for S(−)-ketorolac. Matrix effect was found negligible and the method showed good performances in terms of accuracy (89.6–102.7%) and precision (1.7–6.7%) for both enantiomers. Extraction recoveries of R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac were 82.04, 70.94, and 93.90%, respectively. Results of all stability exercises in human plasma were within acceptable limits. The method was successfully applied to a single dose cross over bioequivalence study in healthy human male volunteers. Incurred Sample Reanalysis (ISR) was performed by randomly selecting 10% of total subject samples of the study using Statistical Analysis Software (SAS). Values of 91.1% for R (+)-ketorolac and 83.5% for S(−)-ketorolac indicated good acceptance for ISR.

1. Introduction

Ketorolac tromethamine is a potent nonnarcotic analgesic compound with cyclooxygenase inhibitory activity which has been developed for oral and parenteral use [1]. The drug is available as a racemic mixture of an equimolar ratio of R(+) and S(−) stereoisomers [2]. Ketorolac ((rac)-5-benzoyl-1,2-3H-pyrrolo[1,2a]pyrrole-1-carboxylic acid) has a chiral carbon atom located within the pyrrolidine ring and is marketed for clinical use as a racemate [3]. The pharmacokinetics of the two enantiomers of ketorolac is substantially different and hence the pharmacokinetic data should be provided for the separate enantiomers [4]. Pharmacologic activity resides almost exclusively with the S(−) stereoisomer [3].

Various methods have been reported for measurement of ketorolac without differentiating between the enantiomers [5–7]. A few methods using HPLC have been reported for resolution of the ketorolac enantiomers [8–15]. However, LC-MS/MS is a promising tool for analysis of drugs in biological matrix. A method described by Ing-Lorenzini et al. for determination of ketorolac enantiomers, however, suffers from a major limitation of involvement of tedious and time consuming extraction procedure using liquid-liquid extraction (LLE) [4]. Achieving better recoveries,

cleaner extracts, requirement of less time for extraction, selective removal of interferences and matrix components, lower requirement for solvent, and avoiding of formation of emulsions are advantages of solid phase extraction (SPE) over LLE. One primary advantage is that SPE renders itself easily to automation. To our knowledge, no entirely validated LC-MS/MS method has been reported in the literature for quantification of ketorolac enantiomers in biological samples.

The overall aim was to develop a sensitive LC-MS/MS method for simultaneous determination of ketorolac enantiomers in human plasma samples of a bioequivalence study. The present method, as detailed in sections that follow, offers a relatively simple sample preparation procedure using SPE and has been comprehensively validated, offering the advantage of simplicity with adequate sensitivity, selectivity, and precision to determine ketorolac enantiomers in plasma samples. Compared to the LLE method described by Ing-Lorenzini et al. the present method is having advantage of less extraction time and better sample quality due to SPE. Following validation, this assay was successfully applied to a bioequivalence study of ketorolac tromethamine to quantify its enantiomers.

2. Experimental

2.1. Chemicals. R(+)-ketorolac (98.09% pure), S(−)-ketorolac (99.28% pure), and S(+)-etodolac (internal standard; 99.23% pure) used to prepare stock solutions were purchased from Varda Biotech (P) Ltd., India. Ammonium formate was purchased from Fluka (Buchs, Switzerland). Formic acid (85% pure) and methanol (99.8% pure) was procured from Fischer Scientific (India). HPLC grade acetonitrile from Spectrochem (India) was used for the preparation of mobile phase. Water was procured in-house using a Milli-Q device (Millipore, Moscheim Cedex, France). K3 EDTA (Ethylene diamine tetra acetic acid) containing plasma batches was obtained from Yash Laboratories, Pune, India. Figure 1 shows the structure of R(+)-Ketorolac, S(−)-Ketorolac, and S(+)-etodolac.

2.2. Instrumentation. The instrumentation consisted of a modular HPLC (Shimadzu, Kyoto, Japan) coupled to AB Sciex API-3200 mass spectrometer (Applied Biosystems, Ontario, Canada), equipped with an electrospray ion interface. The HPLC system consisted of two LC-20AD pumps (identified as pumps A and B), a CTO-20A column oven, a SIL-HTc autosampler, 20A semi-micromixer having mixing volume 100 μl, and DGU-20A$_3$ degasser. The column oven was maintained at 35°C ± 1.0°C.

During method development, chromatographic resolution was optimized on a Chiral-AGP (100 × 4.0 mm, 5 μm) column. A gradient time program (Table 1) was employed. Mobile phase "A" contained ammonium formate buffer (10 mM; pH 4.70): acetonitrile (85 : 15, v/v) whereas mobile phase "B" contained ammonium formate buffer (10 mM; pH 4.70): acetonitrile (70 : 30, v/v). Pump-A was used for Mobile phase A and Pump-B used for mobile phase B. HPLC cycle time is 20 minutes. From time 0.01 to 8.00 min % of mobile phase A is 100% and flow rate is 0.5 ml/min, at 9.00 min % of mobile phase B is 100% and flow rate is 0.5 ml/min, at 9.50 min % of mobile phase B is 100% and flow rate is 0.8 ml/min, at 14.00 min % of mobile phase A is 100% and flow rate is 0.5 ml/min, and at 19.90 min % of mobile phase B is 100% and flow rate is 0.5 ml/min. In the above gradient time program the column equilibration time is from 14.00 min to 20.00 min.

Table 1: Gradient Time Program.

Time (min)	Events		Flow (ml/min)
	% Mobile phase A	% Mobile phase B	
0.01	100	0	0.5
8.00	100	0	0.5
9.00	0	100	0.5
9.50	0	100	0.8
13.50	0	100	0.8
14.00	100	0	0.8
19.80	100	0	0.8
19.90	100	0	0.5
20.00	STOP		

Table 2: Optimized ion source and compound parameters.

Parameter	R(+)-Ketorolac	S(−)-Ketorolac	S(+)-Etodolac
Declustering potential	41.00 V	41.00 V	32.00 V
Entrance potential	10.00 V	10.00 V	10.00 V
Collision energy	25.00 V	25.00 V	19.00 V
Clustering cell exit potential	2.00 V	2.00 V	2.00 V
Temperature	550°C	550°C	550°C
Collision associated dissociation	5	5	5
Curtain gas	25	25	25
Ion spray voltage	1500	1500	1500

The mass spectrometer was operated in positive turbo-ion spray mode. Multiple Reaction Monitoring (MRM) mode was used to monitor *m/z* transitions of 256.2 → 105.3, 256.1 → 105.2, and 288.2 → 172.2, for R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac, respectively. Optimized MRM parameters (ion source and compound parameters) were summarized in Table 2.

2.3. Data Processing. Chromatograms were acquired using Analyst software (version 1.4.1, Applied Biosystems, Ontario, Canada). A calibration curve is constructed using peak area ratios (PARs) of the calibration standards by applying linear, 1/concentration squared weighted, least squares regression algorithm. All concentrations are then calculated from their PARs against the calibration line.

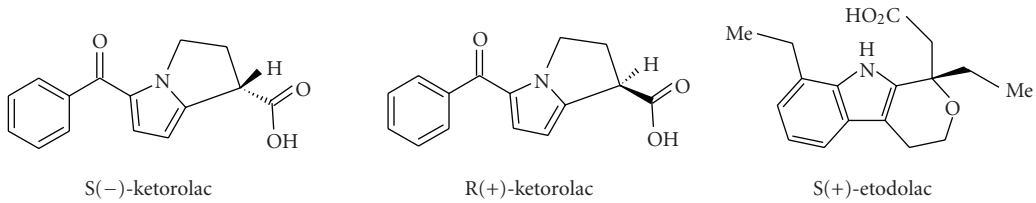


Figure 1: Structures of R(+)-ketorolac, S(−)-ketorolac, and S(+)-Etodolac.

2.4. Stock Solutions. Separate stock solutions of R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac were prepared by dissolving accurately weighed standards in methanol to yield the concentrations of 989021.85 ng/ml,1103318.50 ng/ml, and 110292.61 ng/ml, respectively, and stored in polypropylene container. The concentrations were corrected for purity, moisture content and amount weighed as per certificates of analysis.

2.5. Calibration Standards and Quality Control Samples. Working solutions for calibration curve (CC) standards and quality control samples (QC) were prepared in methanol: water (50 : 50, v/v). The stock solutions of R(+)-ketorolac and S(−)-ketorolac were used to prepare working solutions. These dilutions were spiked in K3 EDTA plasma. Each calibration curve consisted of one blank sample, one blank sample fortified with IS, and eight calibration points ranging from 9.36 to 1198.69 ng/ml for R(+)-ketorolac and from 6.07 to 776.74 ng/ml for S(−)-ketorolac. The QC samples spiked independent of CC standards comprised Lower Limit of Quantification (LLOQ), Low-Quality Control (LQC), Middle-Quality Control (MQC), and High-Quality Control (HQC). Aliquots of the CC and QC were stored below −50°C. Figure 2 shows the representative calibration curves of R(+)-ketorolac and S(−)-ketorolac.

2.6. Biological Sample Preparation. Plasma samples frozen at −50°C were thawed on the day of extraction at room temperature followed by vortexing to ensure homogeneity. IS dilution (50 μl of 2500.00 ng/ml) was added to 300 μl plasma sample and vortexed. Samples were then pretreated with 100 μl of 5% formic acid solution (v/v) and vortexed again. Extraction was carried out using solid phase extraction cartridges (Oasis, HLB 30 mg/1cc, Waters Corporation, USA), preconditioned with 1 ml of methanol followed by 1 ml of Milli-Q water. The cartridges were washed with 1 ml of Milli-Q water followed by 1 ml of 5% methanol (v/v). The elution was carried out twice with 1 ml methanol. The eluent was dried at 50°C using nitrogen gas. The dried residue was reconstituted in 400 μl of solution containing ammonium acetate buffer (10 mM, pH 4.70): acetonitrile (85 : 15, v/v). Ten microlitres of each sample were injected into the LC-MS/MS for analysis.

2.7. Validation. All validation exercises were conducted in conformance to in-house standard operating procedures and the U.S. FDA Guidance document [16]. The results of validation parameters were summarized in Table 3.

3. Results and Discussion

3.1. Selection of Column and Optimization of Chromatographic Conditions. The objective of this study was to chromatographically resolve enantiomers of ketorolac to enable accurate quantification. Two chiral analytical columns, namely, Chirobiotic V2 (25 cm × 4.6 mm, 5 μ) and Chiral AGP (100 × 4.0 mm, 5 μm), were shortlisted during method development.

Baseline chromatographic resolution could not be achieved on the Chirobiotic V2 column with a mobile phase ammonium formate buffer (10 mM, pH 4.00–5.50): acetonitrile (70 : 30, v/v). However, a good resolution was observed on the Chiral AGP column using ammonium formate buffer (10 mM, pH 4.70): acetonitrile.

In order to undertake successful quantification of enantiomers, tuning parameters for ESI+ were optimized for the protonated precursor and product ions of analytes and IS. R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac (IS) were found to have retention time of 5.10 ± 0.71, 4.50 ± 0.71, and 11.50 ± 0.71 min, respectively, under the chromatographic conditions described. Figure 3 shows chromatograms of drug-free plasma, drug-free plasma fortified with internal standard, and limit of quantification sample.

3.2. Linearity, Limit of Quantification, Accuracy, Precision, and Recovery. Linearity of R(+)-ketorolac and S(−)-ketorolac determination was established over a concentration range of 9.36–1198.69 ng/ml and 6.07–776.74 ng/ml, respectively, in spiked human plasma. The selected standard calibration range covered the therapeutic levels of drug in human plasma samples. Linear coefficient of regression ($r > 0.99$) was obtained using least squares linear regression model using peak area ratios. The limit of quantification in the present method was 9.37 ng/ml and 6.09 ng/ml for R(+)-ketorolac and S(−)-ketorolac, respectively.

Three precision and accuracy batches were run to check intra- and interday precision and accuracy. The results for precision and accuracy are summarized in Table 4. The intraday precision ranged from 2.4 to 3.5% and 1.7 to 4.1% for R(+)-ketorolac and S(−)-ketorolac, respectively. The interday precision ranged from 4.1 to 6.3% for R(+)-ketorolac and was from 4.7 to 6.7% for S(−)-ketorolac. The intraday accuracy ranged from 97.7 to 102.6% and 89.6 to 97.7% for R(+)-ketorolac and S(−)-ketorolac, respectively, whereas the interday accuracy ranged from 98.4 to 102.7% for R(+)-ketorolac and from 91.5 to 97.0% for S(−)-ketorolac.

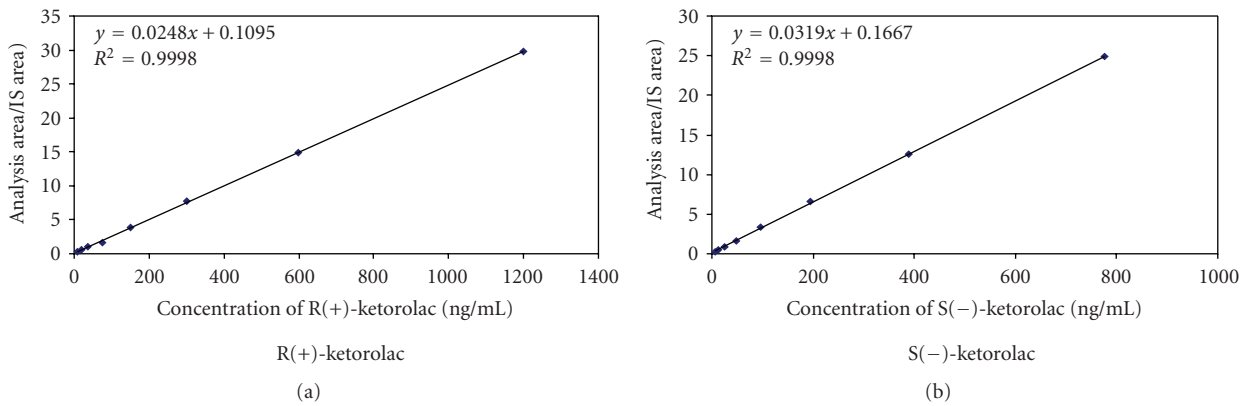


Figure 2: Representative calibration curves R(+)-ketorolac and S(−)-ketorolac.

Table 3: Result of validation parameters.

Validation parameter	R(+)-Ketorolac	S(−)-Ketorolac
Internal standard	S(+)-Etodolac	S(+)-Etodolac
Bench top stability (in plasma samples)	9.02 hr at room temperature	9.02 hr at room temperature
Autosampler stability	110.67 hr at 10°C in propylene container.	110.67 hr at 10°C in propylene container.
Stock solution stability of the drug stored at refrigerated temperature between 1–10°C	11 days	11 days
Carry over effect in matrix	0% at RT of analyte and IS	0% at RT of analyte and IS
Stock stress stability in aqueous dilutions	8.95 hr at room temperature under low light condition in poly propylene container.	8.95 hr at room temperature under low light condition in poly propylene container.
Recovery	82.04%	70.94%
Freeze thaw stability	3 cycle	3 cycle
Dilution integrity	Dilution at 2 times and 4 times	Dilution at 2 times and 4 times
Limit of quantitation (ng/ml)	9.36	6.07
Analytical range (ng/ml)	9.36–1198.69	6.07–776.74
Long-term stability-1	117 days at temperature below −50°C	117 days at temperature below −50°C
Long-term stability-2	283 days at temperature below −50°C	283 days at temperature below −50°C

The recovery/ for R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac was calculated by comparing the peak areas of processed plasma which was prespiked with analytes at low, medium, and high concentration levels, with peak area of aqueous mixture of analytes representing 100% extraction of samples at low, medium, and high concentration levels. The mean extraction recovery of R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac was 82.04, 70.94, and 93.9%, respectively.

3.3. Selectivity. Selectivity was performed using nine different lots of K3-EDTA plasma. From each lot, a single aliquot was processed along with six aliquots of the lower limit of quantification sample spiked in K3-EDTA plasma. There was no significant interference observed at the retention times of analytes and internal standard.

3.4. Dilution Integrity. The samples for dilution integrity were spiked at a concentration approximately two times the concentration of 90% ULOQ (upper limit of quantification). An appropriate volume of this sample was diluted 2-fold and 4-fold using blank matrix. Six replicates at each dilution level were analyzed. The % Accuracy was found to be 104.1 for R (+)-ketorolac and 113.6 % for S(−) Ketorolac. The Precision was found to be 3.0 for both R(+)-ketorolac and S(−) Ketorolac, respectively.

3.5. Matrix Effect and Matrix Factor. In order to determine the matrix effect, nine lots of drug free plasma were chosen and concentrations equivalent to LQC and HQC levels were spiked in each lot. At each level, samples were processed in duplicate. The value of QC samples was back calculated against the freshly spiked calibration curve. CV of 3.1%–3.3% and 3.4%–3.5% and accuracy between 95.3%–101.9% and 92.7%–99.4% were observed for R(+)-ketorolac and S(−)-ketorolac, respectively.

For the calculation of matrix factor, working solutions of drug and IS were prepared at concentrations representing 100% extraction of QC samples at low, middle, and high

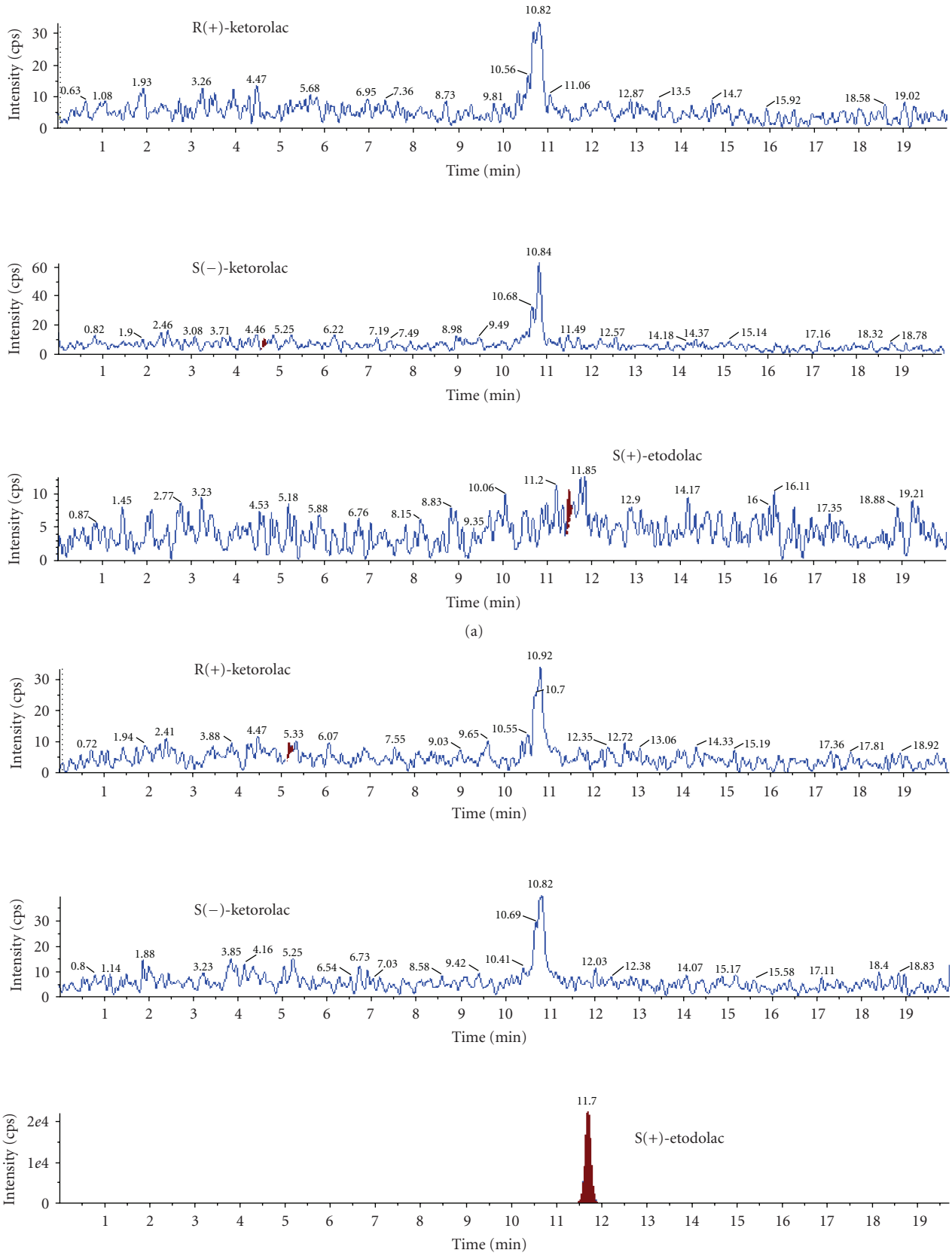
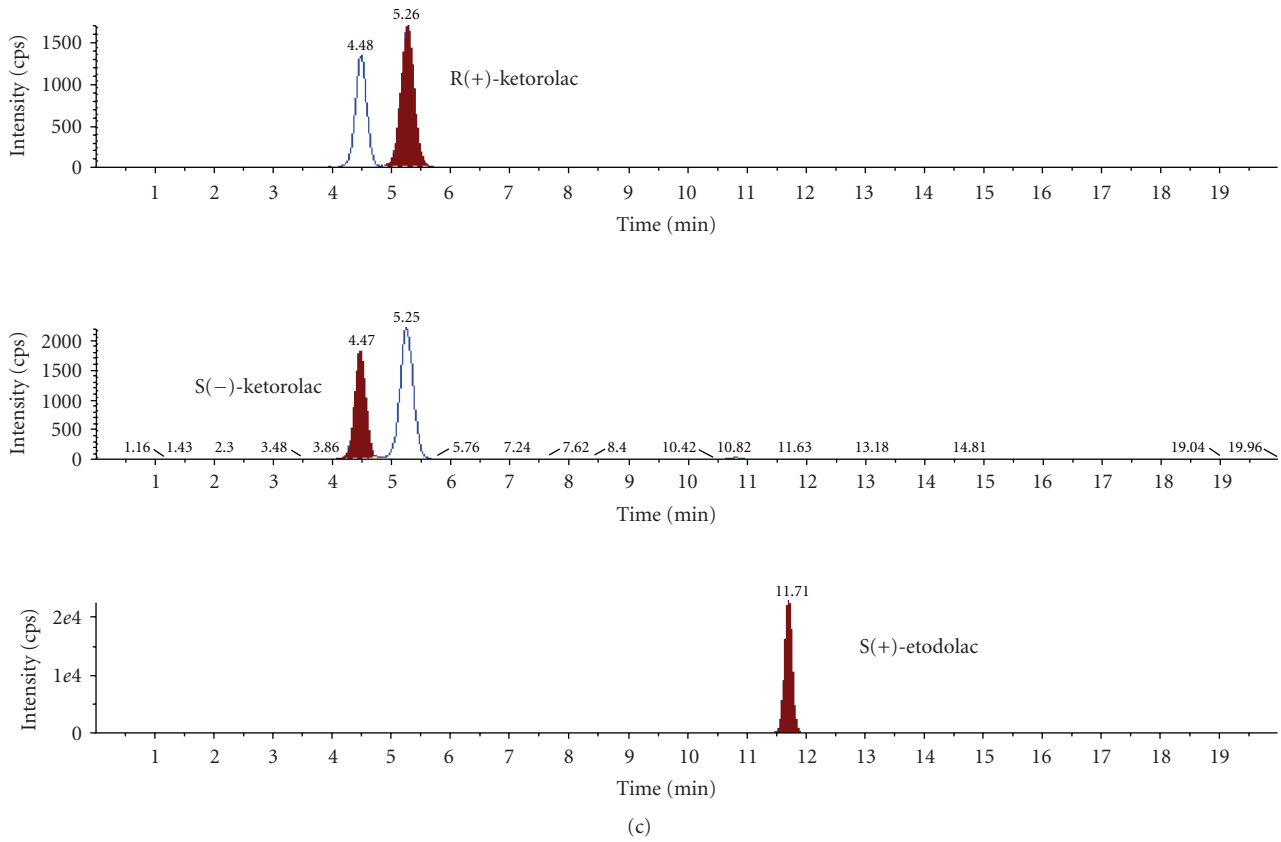


Figure 3: Continued.



(c)

Figure 3: (a), (b), and (c) represent chromatograms of drug-free plasma, drug-free plasma fortified with internal standard, and limit of quantification samples, respectively.

concentrations (aqueous samples). Four replicates each of working solutions of drug and IS at Low, Middle, and High QC levels were run from the same respective vial at each QC level (Total 12 samples). Thereafter, twelve aliquots of screened matrix lots were taken and processed. Every four aliquots were reconstituted with working solutions of drug and IS at Low, Middle and High QC levels respectively. Matrix factor (MF) was calculated at low, middle, and high QC levels by comparing the mean peak area ratio of matrix samples reconstituted with aqueous dilution to that of mean peak area ratio of aqueous samples. The matrix factor of R(+)-ketorolac ranged from 0.97 to 1.01 and for S(−)-ketorolac ranged from 0.96 to 1.00. These values are indicative of neither any ion-suppression nor ion-enhancement. The CV of matrix factor between low middle and high QC levels was found to be 2.5 and 2.7% for R(+)-ketorolac and S(−)-ketorolac, respectively.

3.6. Stock Solution Stability. Stock solution stability of R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac was determined for 11 days. The stocks were stored in a refrigerator between 1 and 10°C. After the stability period, fresh stocks of R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac were prepared. A dilution with same concentration from each of the stocks was prepared and six replicate injections were given. Mean response obtained from stability stock was compared to response of the fresh stock ($n = 6$). The values (% CV) of 102.1 (0.5), 101.5 (0.5), and 102.8% (1.7), respectively, for R(+)-ketorolac, S(−)-ketorolac, and S(+)-etodolac indicated a good stock solution stability for the period evaluated.

3.7. Freeze Thaw Stability, Bench Top Stability, Autosampler Stability, and Long-Term Stability. The results of freeze thaw stability, bench top stability, autosampler stability, and long-term stability are summarized in Table 5. Freeze-thaw stability was assessed by assaying four replicates of QC samples at low concentrations (LQC) and high concentrations (HQC) previously frozen and thawed at room temperature over three cycles. The comparison was made to freshly spiked calibration standards. R(+)-ketorolac and S(−)-ketorolac proved to be stable in biological samples for three Freezes-and thaw cycles. Bench top stability for R(+)-ketorolac and S(−)-ketorolac in human plasma was established for 9.02 h for which samples were left on bench at room temperature and then processed with freshly spiked CC before analysis. In order to assess autosampler stability, four sets of low and high QC samples were kept in an autosampler in polypropylene container programmed at 10°C and were analyzed after 110.67 hours along with freshly spiked low QC (LQC) and high QC (HQC) samples, and the concentration was calculated against the freshly spiked calibration standards.

Table 4: Intra- and interday precision and accuracy for determination of ketorolac enantiomers in human plasma by LC-MS/MS.

Nominal concentration (ng/ml)	Observed (mean ± S.D.) (ng/ml)	%CV	%Accuracy	Number of observations (n)
Intra-day				
R(+)-ketorolac				
9.37 (LOQQC)	9.43 ± 0.33	3.5	100.7	12
24.57 (LQC)	24.01 ± 0.76	3.2	97.7	12
511.90 (MQC)	525.37 ± 15.52	3.0	102.6	12
984.42 (HQC)	972.57 ± 23.15	2.4	98.8	12
S(−)-ketorolac				
6.09 (LOQQC)	5.95 ± 0.24	4.1	97.7	12
15.95 (LQC)	14.24 ± 0.56	3.9	89.3	12
332.35 (MQC)	313.93 ± 7.57	2.4	94.5	12
639.14 (HQC)	572.93 ± 9.91	1.7	89.6	12
Inter-day				
R(+)-ketorolac				
9.37 (LOQQC)	9.33 ± 0.59	6.3	99.5	18
24.57 (LQC)	24.17 ± 1.00	4.1	98.4	18
511.90 (MQC)	525.95 ± 27.56	5.2	96.9	18
984.42 (HQC)	973.85 ± 46.18	4.7	98.9	18
S(−)-ketorolac				
6.09 (LOQQC)	5.91 ± 0.28	4.7	97.0	18
15.95 (LQC)	14.59 ± 0.97	6.7	91.5	18
332.35 (MQC)	321.98 ± 16.87	5.2	96.9	18
639.14 (HQC)	588.65 ± 32.13	5.5	92.1	18

Moreover, the results of frozen storage on stability indicated that the drug was stable for at least 117 days when kept frozen below −50°C.

3.8. Stock Stress Stability. Appropriate dilutions of analytes, internal standard, and reference dilution were prepared from the respective standard stock solutions and stored for 8.95 hours at room temperature in polypropylene container. After this stability period, fresh dilutions of the analytes, internal standard, and reference dilution were prepared (comparison dilutions) from the same respective standard stock solutions from which the stability dilutions were made. Six replicates each of stability dilutions and comparison dilutions were injected from the same vial. The dilutions were found to be stable for the storage period evaluated with percent stability (%CV) of 103.0 (0.7) and 100.2 (0.6) for R(+)-ketorolac and S(−)-ketorolac, respectively.

3.9. Carry-Over Effect in Matrix. Two blank plasma samples, two LLOQ samples, and two ULOQ plasma samples were processed and injected in the following order. One Blank sample was injected, followed by two LLOQ samples, two ULOQ samples, and one blank sample. Percentage carry-over was calculated with respect to the mean of two LLOQ areas at RT of analyte and IS.

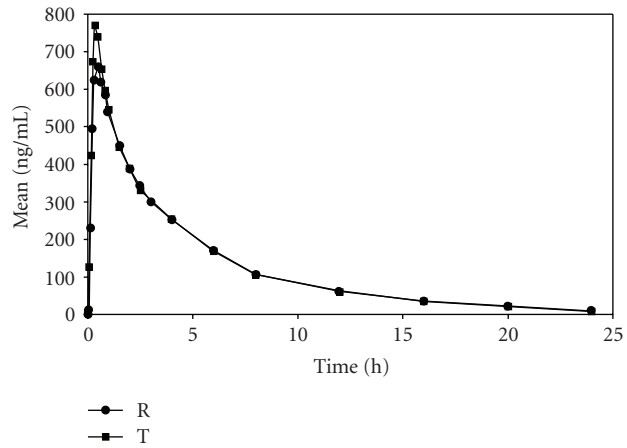


Figure 4: Linear plot of mean plasma R(+)-ketorolac concentration (ng/ml) versus time (h) in healthy adult human male subjects (N = 22).

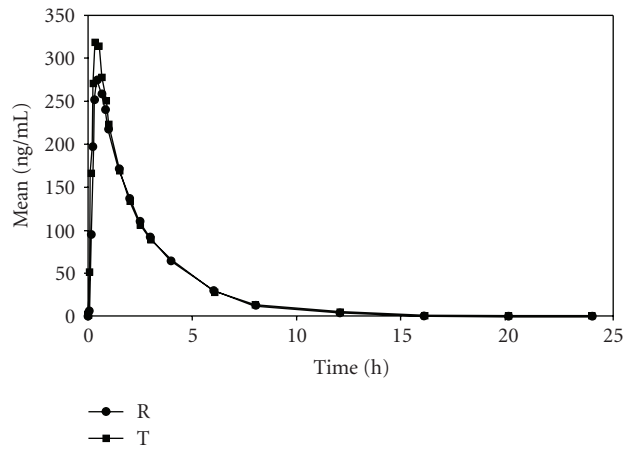


Figure 5: Linear plot of mean plasma S(−)-ketorolac concentration (ng/ml) versus time (h) in healthy adult human male subjects (N = 22).

It was found that there is no carry-over at the RT of analytes and IS.

4. Bioequivalence Study in Healthy Subjects

This method was successfully applied to an open label, balanced, randomized, two-period, two-sequence, single dose, crossover, bioequivalence study of ketorolac involving twenty-two healthy male volunteers following oral administration of 10 mg of ketorolac tromethamine mouth dissolving tablet of Ranbaxy Laboratories Limited (test) under fasting condition and the same was compared with the TORADOL 10 mg tablet (containing 10 mg ketorolac tromethamine) of Roche Products Limited (reference).

All healthy Indian human volunteers were in the age range of 18–45 years, medically examined, and had voluntarily provided their written informed consent before initiation of study. The study protocol and written informed consent

Table 5: Freeze thaw stability, bench top stability, autosampler stability, and long-term stability data of R(+)-ket orolac and S(−)-ketorolac in human Plasma.

Storage period and condition	Analyte	Sample type	Nominal Conc.(ng/mL)	Mean	CV(%)	%Accuracy	%Stability
Freeze thaw cycles(3 cycles)	R(+)-ketorolac	LQC	24.57	24.46	3.2	99.5	101.1
		HQC	984.42	1015.94	3.2	103.2	102.5
	S(−)-ketorolac	LQC	15.95	15.2	3.9	95.3	99.6
		HQC	639.14	625.65	4.4	97.9	101.6
Bench top stability in plasma samples (9.02 hours)	R(+)-ketorolac	LQC	24.57	26.57	3.6	108.2	104.5
		HQC	984.42	959.49	2.5	97.5	101
	S(−)-ketorolac	LQC	15.95	15.77	4.2	98.8	103.7
		HQC	639.14	587.9	2.4	92	102.8
Autosampler stability (110.67 hours)	R(+)-ketorolac	LQC	24.57	23.15	3.7	94.2	97.5
		HQC	984.42	949.18	1.2	96.4	99
	S(−)-ketorolac	LQC	15.95	14.19	3.9	88.9	97
		HQC	639.14	571.73	1.8	89.5	97.2
Long term stability 1 (117 Days)	R(+)-ketorolac	LQC	24.57	26.84	2.9	109.2	113
		HQC	984.42	1034.84	1.2	105.1	99.5
	S(−)-ketorolac	LQC	15.95	17.39	3.7	109	109.9
		HQC	639.14	665.13	1.1	104.1	99.1
Long term stability 2 (283 Days)	R(+)-ketorolac	LQC	24.57	25.72	1.2	104.7	101.3
		HQC	984.42	977.32	2.4	99.3	99
	S(−)-ketorolac	LQC	15.95	17.39	1.1	104.5	101.2
		HQC	639.14	665.13	2.8	98.6	100

were approved by the ethical committee of Jamia Hamdard Institutional Review Board, New Delhi, India.

Venous blood samples were collected in K3 EDTA tubes predose and at 0.083, 0.167, 0.250, 0.333, 0.500, 0.667, 0.833, 1.000, 1.500, 2.000, 2.500, 3.000, 4.000, 6.000, 8.000, 12.000, 16.000, 20.000, and 24.000 h after dosing. Plasma was separated by centrifugation and the separated plasma samples were stored below −50°C until analysis. Following analysis, pharmacokinetic parameters were calculated by noncompartmental analysis using WinNonlin Professional software (Version 5.0, Pharsight Corp., Mountain View, CA, USA). The peak plasma concentration ($C_{\max}$) and time ($T_{\max}$) to reach $C_{\max}$ were read directly from the data. The total areas under the plasma concentration-time curve from time zero to infinity ($AUC_{0\to\infty}$) and from time zero to the last measurable concentration ($AUC_{0\to t}$) were calculated using the linear trapezoidal rule-extrapolation method. Mean plasma concentration–time profiles of R(+)-ketorolac and S(−)-ketorolac are shown in Figures 4 and 5 and the mean estimates of pharmacokinetic parameters derived from the plasma concentration profiles are summarized in Table 6.

The mean Cmax of R(+)-ketorolac was 776.25 ± 245.57 ng/ml and 808.54 ± 200.22 ng/ml, respectively, for reference and test products, whereas the mean Cmax for S(−)-ketorolac was 319.09 ± 107.76 for the reference product and 338.50 ± 91.77 ng/ml for the test product.

An Incurred Sample Reanalysis (ISR) was performed on 90 sample points from 15 different subjects selected randomly using statistical analysis software (SAS). Three time points from each period of these identified 15 subjects were selected for ISR. One sample was at the $T_{\max}$, second sample was at the time point at which the concentration was at least threefold of LOQ, and third sample was the preceding time point of threefold of LOQ. The results, as summarized in Table 7, clearly demonstrate that for each analyte the difference between the original and reanalyzed values was within 20% for at least 67% of the total samples reanalyzed [17]. Furthermore, Bland-Altman plots were generated individually for both analytes (Figures 6 and 7). For R(+)-ketorolac, statistical analysis of this data revealed a mean ratio (MR) of 0.97; ratio limits (RL) from 0.95 to 0.99, not including 1 (acceptance range, 0.83–1.20); and limits of agreement (LA) from 0.86 to 1.08 (acceptance range, 0.83–1.20). The acceptance ranges for both the RL as well as the LA were met. The fact that the RL did not quite include 1 tends to suggest a very slight bias to the data. As can be seen upon inspection of the percentage differences for R(+)-Ketorolc, 42 of the percentage differences calculated are negative compared with 48 that were positive. Similarly, for S(−)-ketorolac, statistical analysis of the data revealed a mean ratio (MR) of 0.93; ratio limits (RL) from 0.91 to 0.95, not including 1 (acceptance range, 0.83–1.20); and limits of agreement (LA) from 0.84 to 1.04 (acceptance range, 0.83–1.20). The acceptance ranges for both the RL as well as the LA were met for S(−)-ketorolac too. For the percentage differences as observed for S(−)-Ketorolac, 28 of the percentage differences calculated are negative compared with 62 that were positive. The results of this analysis are presented in

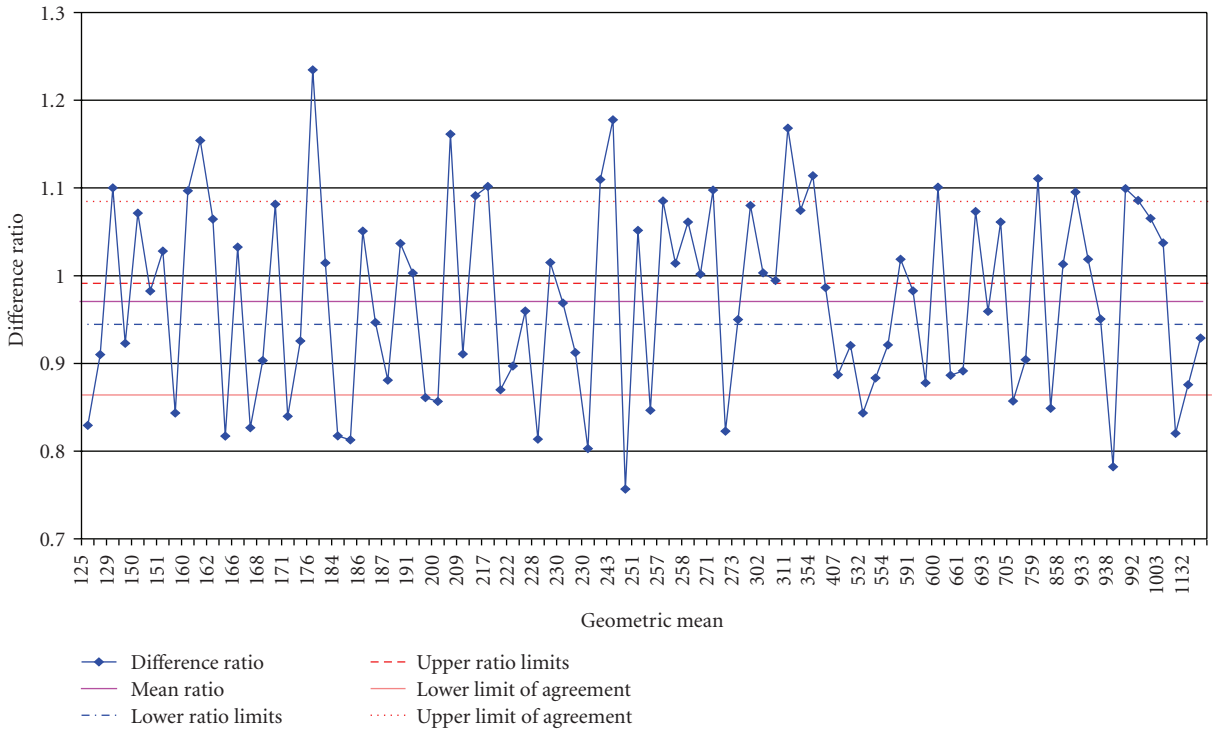


Figure 6: Bland-Altman plot of the data obtained from ISR of R(+)-ketorolac.

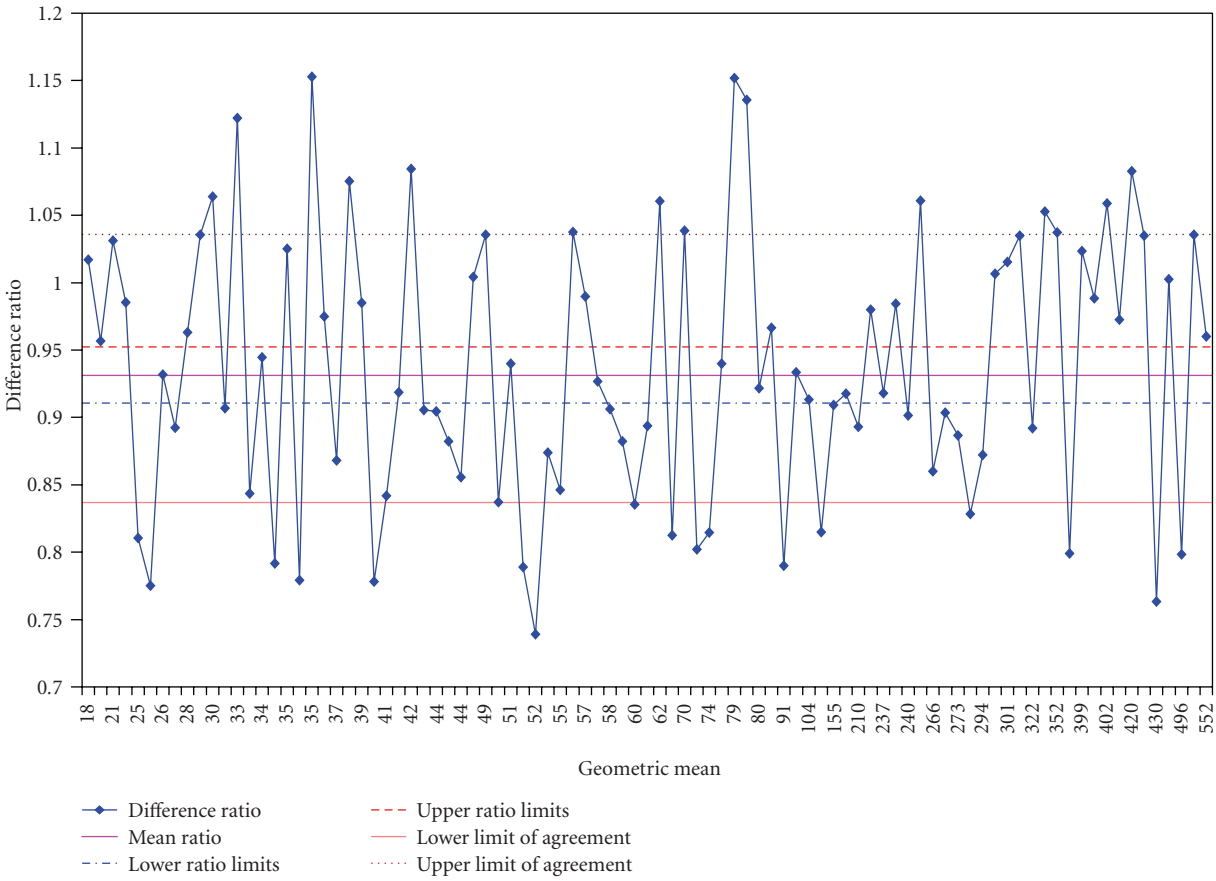


Figure 7: Bland-Altman plot of the data obtained from ISR of S(−)-ketorolac.

Table 6: Pharmacokinetic parameters (mean ± SD) of ketorolac tromethamine mouth dissolving tablets (10 mg) based on its plasma concentrations.

Parameters ($n = 22$)	Tmax (h)		Cmax (ng/ml)		$AUC_{0\to t}$ (h ng/ml)		$AUC_{0\to\infty}$ (h ng/ml)	
	R	T	R	T	R	T	R	T
R(+)-Ketorolac	0.52 ± 0.27	0.37 ± 0.10	776.25 ± 245.57	808.54 ± 200.22	2962.76 ± 800.14	3042.10 ± 850.23	3085.31 ± 821.83	3163.37 ± 885.42
S(−)-Ketorolac	0.54 ± 0.26	0.41 ± 0.11	319.09 ± 107.76	338.50 ± 91.77	725.72 ± 215.97	755.04 ± 234.56	750.13 ± 218.67	779.67 ± 236.07

R: Reference product; T: Test product; values represented as mean ± S.D.

Table 7: Confirmatory incurred sample reanalysis of R(+)-ketorolac and S(−)-ketorolac.

	R(+)-Ketorolac	S(−)-Ketorolac
No. of total samples taken for ISR	90	90
No. of samples meeting the acceptance criteria (i.e., % difference between original and reanalyzed value must be within 20%)	82	76
% of samples meeting the acceptance criteria	91.1%	84.4%

Figures 6 and 7. Result of ISR is summarized in Table 7. For R(+)-ketorolac 91.1% and for S(−)-ketorolac 84.4% of total reanalyzed samples were meeting the acceptance criteria showing a good reproducibility of the method.

5. Conclusion

A simple and accurate chiral LC-MS/MS method was described for the enantiomeric separation of ketorolac. Chiral AGP, a α1-acid glycoprotein-based column was found to be selective for the enantiomers of ketorolac. Method validation was carried out using Chiral AGP column due to better chromatographic results achieved on the column. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability-indicating and was used successfully for a bioequivalence study of 10 mg of ketorolac tromethamine mouth dissolving tablet.

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