RESEARCH PAPER | Science | Volume : 4 | Issue : 8 | August 2014 | ISSN - 2249-555X

A Simple, Rapid, Sensitive and Specific Liquid Chromatography Tandem Mass Spectrometric Method Development and Validation for the Quantification of S-(+) And R-(-) Etodolac in Human Plasma

KEYWORDS: R-(-) Etodolac & S-(+) Etodolac, S-Etodolac D4, LC-MS/MS, human plasma, validation

Dr. Tushar Barot
Research Scientist, Famy care Ltd, Pharmasez, Ahmedabad, Gujarat, INDIA.

Hardik Brahmbhatt
Lecturer, Shivrath center of Excellence in Clinical Research affiliated with Gujarat University, Ahmedabad, Gujarat, INDIA.

ABSTRACT *A stereo selective liquid chromatography tandem mass spectrometry method was developed using solid phase extraction (SPE) for quantification of R-(-) Etodolac & S-(+) Etodolac in human plasma using S-Etodolac D4 as internal standard. The separation was achieved by chiral-AGP (100×4mm) 5µm column and 10mM Ammonium acetate (pH 6.0): Acetonitrile (75:25 V/V) as mobile phase, at flow rate 0.500 mL/min. The ion mode was positive. The detection was carried out by Q3 mass detector. The retention time for R-(-) Etodolac & S-(+) Etodolac were found to be 1.90min and 2.57min respectively and 2.50min for S-Etodolac D4. The total run time of S-(+) Etodolac and R-(-) Etodolac was 4.2 minute. The limit of quantification (LLOQ) of R-(-) Etodolac & S-(+) Etodolac were obtained 0.15 µg/mL and 0.10µg/mL respectively using chiral-AGP (100×4mm) 5µm column and using Q3 Mass detector.*

1. Introduction:

The anti-inflammatory analgesics popularly known as Non-narcotic analgesic agents are also associated with anti-pyretic property. Non-steroidal anti-inflammatory drugs (NSAIDs) are used in humans and domestic animals due to their anti-inflammatory, analgesic and anti-pyretic effects [1]. Etodolac (1,8-diethyl-1,3,4,9 tetrahydropyrano[3,4-b]-indole-1-acetic acid) possesses an indole ring as the aryl portion of NSAID drugs and indicated for the use in acute and Long-term management of pain and Osteoarthritis [2]. In patient with Osteoarthritis, 200-300mg twice a daily given for better effect [3].

Earlier publications have described methods of analyzing Etodolac in biological samples. The techniques adopted include; spectrometry and spectrofluorimetry [4], gas chromatography (GC) [5], high performance liquid chromatography (HPLC) [6,7] and gas chromatography–mass spectrometry (GC–MS) [8]. However, these methods suffer from a number of disadvantages, a large volume of plasma (≥0.02 mL) [5–8], lengthy run times (>3 min) [5–8], the lack of an internal standard (IS) [6-7] and lower sensitivity, which can more accurate analysis (0.1µg/mL) [6-8]. And liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) system with a 100 ng/mL lower limit of quantification (LLOQ) and a 3 min run time [9].

The main goal of this study was to develop stereo selective liquid chromatography tandem mass spectrometry of R-(-) Etodolac & S-(+) Etodolac in human plasma with total run time of S-(+) Etodolac and R-(-) Etodolac was 4.2 minute and limit of quantification (LLOQ) of R-(-) Etodolac & S-(+) Etodolac were obtained 0.15 g/ml and 0.10µg/ml respectively.

2. Experimental

2.1 Materials

R-(-) Etodolac, S-(+) Etodolac, S-Etodolac D4 and Dimethyl Sulfoxide (DMSO) obtained from Fleming Laboratories Ltd (Hyderabad, India) and Methanol and acetonitrile (HPLC grade) from J.T. Baker (Philipsburg, NJ, USA).Water was purified using a Milli-Q purification system (Millipore Corp., Bedford, MA, USA). All other chemicals and solvent were of the highest analytical grades available.

2.2 Calibration Standard and Quality Control Samples

A stock solution of R-(-) etodolac, S-(+) Etodolac, S-Etodolac D4 was prepared by Weighing approximately 25.0 mg of R-(-) Etodolac,10.0 mg S-(+) Etodolac and 5.0 mg S-Etodolac D4 working / reference standard respectively ,transfer it into 10.0ml volumetric flask and Dissolve it 50µl DMSO and make up the volume with methanol. Calibration curve of etodolac prepared for R-(-) Etodolac and S-(+) Etodolac by spiking 49.990, 44.990, 37.439, 24.935, 7.480, 2.498, 0.874, 0.437and 8.751, 7.876, 6.553, 4.304, 1.309, 0.437, 0.153, 0.077 µg/mL respectively. Quality control sample (QC) for R-(-) etodolac and S-(+) Etodolac was 49.990, 40.990, 24.600, 3.440, 1.033, 0.465 and 8.751, 7.176, 4.306, 0.603, 0.181, 0.081µg/mL in plasma.

2.3 Chromatographic Conditions and Instrumentation

A Waters Acquity UPLC system and a Waters Micromass QuattroPremier triple quadrupole mass spectrometer equipped with a turbo electrospray interface in positive ionization mode (Waters Ltd., Watford, UK) had been used for LC-MS/MS analysis. Positive ion mode were used to detect R-(-) Etodolac, S-(+) Etodolac and Internal Standard. The analytical column used was Chiral AGP, 100×4mm, 5µm (Daicel chemical industries Ltd, France). Mobile phase in Line A was 25 mL Acetonitrile and in Line B was 10mM Ammonium acetate buffer pH 6.0. Sample analysis was done by maintaining flow rate of 0.500 mL/min.

2.4 Sample Preparation

Plasma samples were stored at -70^{0}C and allowed to thaw at room temperature through vertex it. Transfered the required quantity of CC/QC samples (0.500mL)along with subject samples (0.500mL), added 25 µl ISTD (51.0 µg/mL) and also 5% of formic acid. Conditioned the Starta – X polymeric sorbent cartridge with 1mL of methanol and 1mL of water, with the Solid phase extraction method (SPE), sample was loaded and washed with 2 mL of water. Sample were eluted with 2 mL of methanol and evaporated to dryness at 50^{0}C. The reconstitute solution 1 mL, were added to dry vial and solution 10µl injected in LC-MS/MS.

2.7 Method Validation

The method was validated in terms of precision, accuracy,

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recovery, selectivity, linearity, sensitivity and stability according to the guidelines issued by the food and drug administration (FDA) for the validation of bioanalytical methods [10].

2.7.1 Accuracy and Precision

Coefficient of variance (CV),used to determined Intra and Inter day assay precision and intra- and inter-day assay accuracies were expressed as percentages of the theoretical concentration, as accuracy (%) = (found concentration/ theoretical concentration)×100. Intra-day assays were performed using five replicates during 1 day and inter-day assays were performed on four separate days. FDA Recommended that, acceptance criterion for each back-calculated standard concentration was a 15% deviation from the normal value except at the LLOQ, which was set at 20% [10].

2.7.2 Recovery and Selectivity

Specificity was evaluated by using 8 batches of blank Human plasma including Hemolytic and lipimic plasma. It was tested for the presence of endogenous compounds that might interfere with analyte, using the Solid Phase Extraction procedure and chromatographic conditions, and results were then compared with those obtained with a solution of the analyte at a concentration near the LLOQ. The Absolute recovery and Absolute matrix effect were calculated by using pre extraction and post extraction peak level. The recovery calculated in amount of percentage.

2.7.3 Linearity and sensitivity

A calibration curve was prepared using a double-blank sample (a plasma sample without etodolac and Intenal Standard) and seven calibration samples covering the whole range (0.1–25 µg/mL) by the peak area ratio of etodolac against Intenal standard. Concentrations of R-(-) etodolac and S-(+) etodolac were calculated from these area ratios using the calibration curve. The linearity was calculated as a correlation coefficient (r^2) of 0.99 or better was deemed satisfactory.

2.7.3 Stability

The stability of R-(-)etodolac and S-(+) etodolac were calculated at different time and different temperature of different QC samples (HQC,MQC,LQC). Stability test were include (a) freeze–thaw stability, which was determined after three freeze–thaw cycles on consecutive days; (b) bench top stability, which was determined by keeping a plasma on bench for 24hrs in room temperature, (c) Short and long term stock solution stability.

3. Result and discussion

3.1 MS detection and Chromatographic conditions

The major instrumental parameters of the mass spectrometry are summarized in Table 1. Full scan mass spectra and product ion scan spectra of R-(-)etodolac and S-(+) etodolac and IS were obtained by direct infusion into the mass spectrometer at a flow rate of 0.500 mL/min. R-(-)etodolac, S-(+) and IS mass spectra exhibit protonated species [M+H]+ at m/z 288.35>172.12, m/z 288.41>172.12and m/z 292.36>176.10, respectively, which were chosen as precursor ions. The limit of quantification (LLOQ) of R-(-) Etodolac & S-(+) Etodolac were obtained 0.15 g/mL and 0.10µg/mL respectively.

Table 1.
Mass Tune Parameter

Parameter	R-(-) Etodolac	S-(+) Etodolac	S-Etodola-cD4
Capillary (KV)	3.0	3.0	3.0
Source temperature	100°C	100°C	100°C
Desolvation temperature	400°C	400°C	400°C
Cone (V)	14	12	12
LM Resolution 1	11.0	11.0	11.0
HM Resolution 1	11.0	11.0	11.0
Collision energy (eV)	28	26	20
Entrance	2	2	2
Exit	2	2	2
LM Resolution 2	11.0	11.0	11.0
HM Resolution 2	11.0	11.0	11.0
Detection	288.35> 172.12	288.41> 172.12	292.36> 176.10
Dwell time	0.200sec	0.200sec	0.200sec
Desolvation gas flow	800	800	800
Cone gas flow	20	20	20
Ion energy 1	0.5	0.5	0.5
Ion energy 2	0.2	0.2	0.2

3.2 Method validation

3.2.1 Selectivity

Selectivity method was found by using 8 batches of plasma. The fig 1(a),(b) blank human plasma extract; fig 2(a),(b) extract spiked only with IS; fig 3(a),(b) extract spiked with etodolac and IS. As shown in Fig. 1, no endogenous peaks were observed at the retention times of etodolac or IS. Fig. 3 shows MRM chromatograms at the LLOQ (0.1µg/mL and 0.15µg/mL) of the calibration curve.

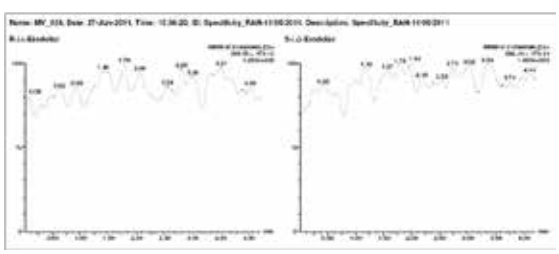

Fig 1(a) blank plasma extract of R-(-)etodolac and S-(+) etodolac

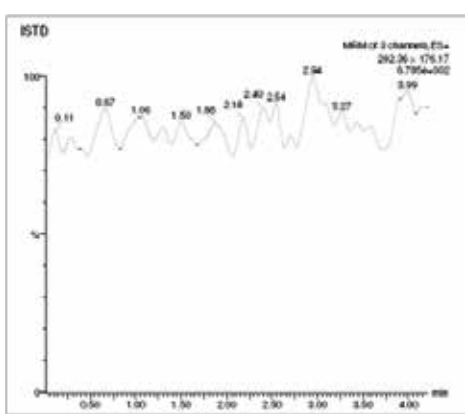

Fig 1(b) blank plasma extract of IS

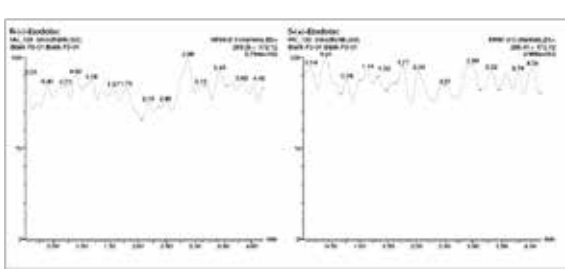

Fig 2 (a) R-(-)etodolac and S-(+) etodolac extract spiked only with IS

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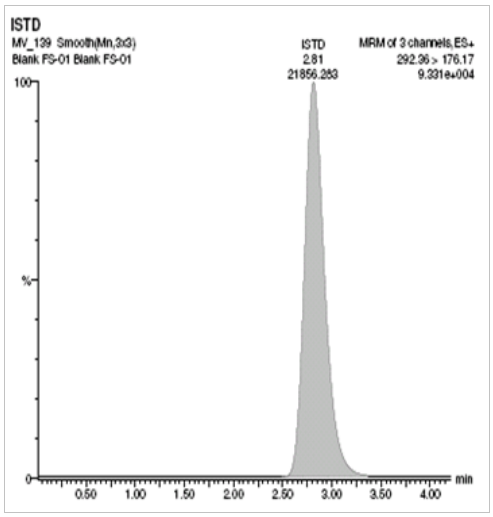

Fig 2(b) IS extract spiked

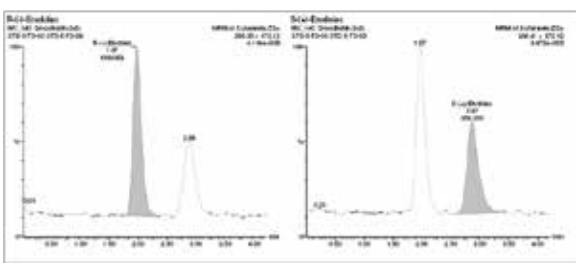

Fig 3(a) Extract spiked with R-(-) etodolac and S-(+) etodolac and IS

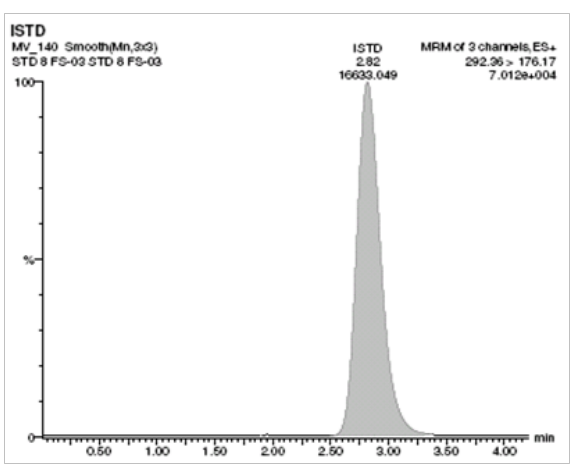

Fig 3(b) Extract Spiked with IS

3.2.2 Linearity and sensitivity

The regression type was 1/(conc)² and peak area ratio for 8 point calibration curve of plasma was found to be linear from 15.006 µg/mL to 0.150µg/mL for R-(-)-Etodolac and 10.002 µg/mL to 0.100 µg/mL S-(+)-Etodolac. The lower limit of qualification were 0.150 µg/ml for R-(-)-Etodolac and 0.100 µg/ml S-(+)-Etodolac. The best run accuracy and precision for R-(-)-Etodolac at 0.150 µg/ml was 103.6% and 3.3% and S-(+)-Etodolac at 0.100 µg/ml as 101.7%and 0.6% respectively.

3.2.3 Accuracy and Precision

The intra day accuracy for R-(-)-Etodolac and S-(+)-Etodolac, the total accuracy of LLOQ, QC. Ranged from 88.1% to 107.4% and 90.8% to 107.8%, which is within the acceptance range of % nominal ± 20%. The inter day accuracy for R-(-)-Etodolac and S-(+)-Etodolac ,the total accuracy of LLOQ, QC are 96.6% and 98.3%, which is within the acceptance range of % nominal ± 20%. The intra day precision For R-(-)-Etodolac and S-(+)-Etodolac, the pression of LLOQ QC range from 6.4% to 16.5% and 4.2% to 14.9%, which is within the acceptance range of %CV ± 20%. The inter day precision For R-(-)-Etodolac and S-(+)-Etodolac, the pression of LLOQ QC range from 14.3 % and 13.1 % , which is within the acceptance range of %CV ± 20%.

3.2.4 Recovery and Matrix effect

The area ratio of analyte with ISTD of extracted low, medium and high QC sample were compared against the respective post extracted quality control samples. Recovery for R-(-)-Etodolac and S-(+)-Etodolac , the total accuracy of high .medium and low QC samples were 102.652%, 94.186%, 95.993% and 103.519% 97.066%, 92.157% respectively.

3.2.5 Stability

Studies were checked to determine drug activity with stabilty in plasma and in the mobile phase used for making solution which used in analysis, and no degradation were seen (data not shown). These results indicate that etodolac was stable under bench (room temperature) Table 2 and freeze–thaw conditions, and importantly no stability-related problems were encountered during routine sample analysis.

Table 2: Bench Top Stability for R-(-)- Etodolac and S-(+)- Etodolac in human plasma

Sr. NO.	R-(-)- Etodolac				S-(+)- Etodolac			
	Freshly Spiked		After 06 : 52 Hours		Freshly Spiked		After 06 : 52 Hours	
	L QC	H QC	L QC	H QC	L QC	H QC	L QC	H QC
	Nominal Conc. (µg/ml)		Nominal Conc. (µg/ml)		Nominal Conc. (µg/ml)		Nominal Conc. (µg/mL)	
	0.325	12.307	0.325	12.307	0.217	8.201	0.217	8.201
1	0.345	12.481	0.371	12.468	0.216	8.451	0.244	8.445
2	0.358	12.394	0.359	12.169	0.227	8.467	0.230	8.574
3	0.359	12.781	0.345	12.886	0.248	8.568	0.220	8.276
4	0.353	13.199	0.384	12.725	0.216	8.276	0.241	8.396
Mean	0.354	12.714	0.365	12.562	0.227	8.441	0.234	8.423
	% Mean ratio		103.110	98.806	% Mean ratio		103.087	99.790
Acceptance Criteria:								
% mean ratio should be ± 15%								

4. Conclusion

A stereo selective liquid chromatography tandem mass spectrometry method for etodolac, based on solid phase extraction method, prove to rapid and sensitive in main portion of stereo selective determination of etodolac in human plasma. The analytical method was found to be valid for the analysis of R-(-)-Etodolac and S-(+)-Etodolac in Human plasma over a range of 15.006 µg/mL to 0.150 µg/mL and 0.150 µg/mL and 10.002 µg/mL to 0.100 µg/mL for R-(-)-Etodolac and S-(+)-Etodolac in Human plasma.

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