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LIQUID CHROMATOGRAPHY/NEGATIVE ION ELECTROSPRAY TANDEM MASS SPECTROMETRY METHOD FOR THE QUANTIFICATION OF ETODOLAC IN HUMAN PLASMA: VALIDATION AND IT'S APPLICATION TO PHARMACOKINETIC STUDIES

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Abstract:

A simple reverse phase liquid chromatographic and mass spectroscopic analytical method has been developed and validated for estimation of Etodolac in plasma. Methods: The separation was carried out on CHIRAL AGP 100x4mm, 5µm connected to guard column as Stationary phase, Mobile Phase: Acetonitrile : 2mM ammonium acetate Elution mode : Isocratic A: B= 85:15% v/v Flow rate: 0.8 ml/min using SPD M-10AVP photo diode array detector at 38.10 nm. Results: The described LC MS method was linear over a concentration range of 0.8-13.0ng/ml. Dimenhydrinate was used as internal standard. The Etodolac and Dimenhydrinate showed retention times of 1.5 and 2.1 respectively. The limit of detection (LOD) and the limit of quantification (LOQ) for etodolac was 0.20 ng/ml, 0.50 ng/ml and for Dimenhydrinate 0.09, 0.21 ng/ml respectively. The stability of the drug spiked human plasma samples during three freeze thaw cycles were stable in plasma for about one month when stored at frozen state. The results of the study showed that the proposed LC MS method is simple, rapid, precise and accurate, which is useful for the estimation of etodolac in bulk fluids and biological plasma sample analyte with accuracy and reproducibility.

Keywords: *Etodolac, LC MS method, Dimenhydrinate and Freeze thaw cycles.*

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INTRODUCTION

Etodolac (ETD) is a nonsteroidal anti-inflammatory drug (NSAID). NSAIDs are used for the management of mild to moderate pain, fever, and inflammation. They work by reducing the levels of prostaglandins, which are chemicals that are responsible for pain and the fever and tenderness that occur with inflammation. Etodolac blocks the enzyme that makes prostaglandins (cyclooxygenase), resulting in lower concentrations of prostaglandins. As a consequence, inflammation, pain and fever are reduced. Post-marketing studies demonstrated that Etodolac inhibition of cyclooxygenase is somewhat COX-2 selective similar to celecoxib and other "COX-2 inhibitors." Unlike rofecoxib, both Etodolac and celecoxib can fully inhibit COX-1 and are designated as having "preferential selectivity" toward COX-2. The (inactive against COX) r-enantiomer of Etodolac inhibits β-catenin levels in hepatomacells [1,2].

Pharmacology & Mechanism Action of Etodolac (ETD)

Etodolac (ETD): NSAID's are used for the management of mild to moderate pain, fever, and inflammation. They work by reducing the levels of prostaglandins, which are chemicals that are responsible for pain and the fever and tenderness that occur with inflammation. Etodolac blocks the enzyme that makes prostaglandins (cyclooxygenase), resulting in lower concentrations of prostaglandins [3-6]. As a consequence, inflammation, pain and fever are reduced.

Effects, contraindications and adverse effects of Etodolac (ETD)

ETD should be avoided by patients with a history of asthma attacks, hives, or other allergic reactions to aspirin or other NSAIDs. Upset stomach, nausea, diarrhoea, drowsiness, or dizziness may occur. Other side effects include: easy bruising/bleeding, difficult/painful swallowing, hearing changes (such as ringing in the ears), mental/mood changes, swelling of the ankles/feet/hands, sudden/unexplained weight gain, change in the amount of urine, unexplained stiff neck, vision changes, unusual tiredness. It also should be avoided by patients with peptic ulcer disease or poor kidney function, since this medication can worsen both conditions. Etodolac is used with caution in patients taking blood thinning medications (anticoagulants), such as warfarin (Coumadin), because it increases the risk of bleeding,

Patients taking both lithium and ETD may develop toxic blood lithium levels. Additionally, Etodolac has been found to interact with certain anti-depressant medications, such as sertraline or fluoxetine, which can increase risks of stroke, heart attack, and other cardiovascular conditions [7-9]. NSAIDs should be discontinued prior to elective surgery because of a mild interference with clotting that is characteristic of this group of medicines. ETD is best discontinued at least four days in advance of surgery [10-13].

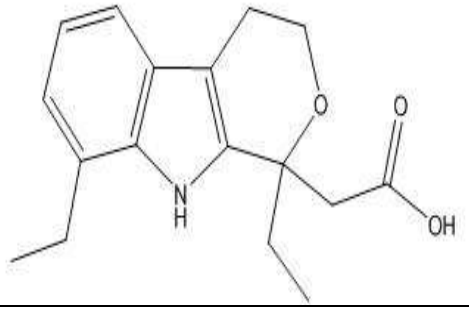
Current therapeutic and clinical uses of Etodolac (ETD)

ETD is used to relieve pain from various conditions. It also reduces pain, swelling, and joint stiffness from arthritis. This medication is known as a nonsteroidal anti-inflammatory drug (NSAID). It works by blocking your body's production of certain natural substances that cause inflammation. Its medication may also be used to treat gout attacks [14-18].

Dosage Regimen

Tablets: 400 & 500 mg, Capsules: 200 & 300 mg

Table 1: Structural features of Etodolac (ETD)

Official Name	Chemical Name(s)	Structure
Etodolac	(RS)2—(1,8-Diethyl-4,9-dihydro-3H-pyrano[3,4-b]indo-1-yl)acetic acid	

The aim of the present study is to achieve more accurate, precise, selective, sensitive and more rapid assay methods for quantification of the following drug and/or their metabolite[s] in human biological plasma. The analytical method provides confidence to use the developed method in a regulatory environment of pharmaceutical industry without any further modification. This makes that the developed analytical method will be use full for the estimation of these drugs in either combined dosage forms or in its pure form [19, 20]. The developed bioanalytical assay method could then be applied to clinical assessment studies to obtain true pharmacokinetic parameters in human biological plasma. HPLC-UV, HPLC-MS, LC-MS/MS, GC-MS and GC-MS/MS methods have been reported. These bioanalytical assay methods use complicated extraction instruments, long and dreary extraction methods, and large amounts of mate trials i.e. solvents, chemicals and/or biological fluids for extraction while other assay methods has a long turn-around time for processing and analysis.

EXPERIMENTAL

Materials and Instrumentation

The Etodolac drug substance was obtained from Creative organics, Hyderabad and Dimendydranite (Internal standard), [IS] was obtained from Triveni chemicals, Vapi as a gift sample. Chemical structure is presented in Table **1.**

HPLC-grade Li-Chrosolv methanol was purchased from Merck (Darmstadt, Germany). The Orochem 30 mg/1cc, DVB-LP cartridges solid-phase extraction (SPE) cartridge (30 mg) was purchased from Waters (Milford, USA) and ammonium acetate was purchased from Merck (Worli, Mumbai, India). HPLC grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC/MS/MS instrument and conditions

The LC-MS/MS was TSQ Endura triple Quadrapole mass spectrometer was used to acquire the data. The chromatography was performed on a Chiral AGP, 100×4 mm, 5µm with guard column (0.4cm Ø × 1 cm 5µm 2-pack) within 6.0 min. The isocratic mobile phase composition was a mixture of 10 mM ammonium acetate: acetonitrile (85:15, v/v), which was pumped at a flow rate of 0.8 mL/min. Quantitation was performed on a triple Quadrapole mass spectrometer employing electro spray ionization technique, operation in selective reaction monitoring (SRM) and positive ion mode. The precursor to product transitions monitored for Etodolac and internal standard were m/z 288.232→171.945 and 292.234→176.021 respectively. The assay method was validated with the linear range of 51.443 ng/mL to 2114.862 ng/mL for S-Etodolac. Data processing was performed on Thermo LCQuan 2.5.6 software.

Table 2: Main working parameters for liquid chromatography

Parameter	Conditions
Column	**CHIRAL AGP 100x4mm, 5µm connected to guard column**
Mobile Phase	**85:15 (10mm Ammonium Acetate pH 6.5: ACN)**
Flow rate	**0.8 mL/min (Split) (50:50)**
Injection Volume	**10.0 µL**
Retention time	**Drug 1 : 2.17 ±.0.8 min (Etodolac)** **ISTD : 1.30 ± 0.8 min (Dimenhydrinate)**
Run time	**3.2 ± 0.5 min.**
Auto sampler temperature	**10°C**
Detector	**Mass**
Column oven temperature	**10 ± 2°C**

Table 3: Main working parameters for tandem mass spectrometry

Scan type	**SRM(MS/MS)**	
Parameters	**DRUG 1**	**ISTD**
Tube lens offset	72	72
Skimmer offset	10	10
Collision energy	15	15
Collision pressure	1.4	
Resolution (Q1&Q3)	0.70	

Preparation of calibration curve (CC) standards and quality control (QC) samples

A stock solution of Etodolac (about 1.2 mg/mL) and IS solution of (2 µg/mL) were prepared in methanol (v/v). Working solutions for calibration and controls were prepared by appropriate dilution in water: methanol (20:80 v/v; diluent). Calibration standards were prepared by spiking blank plasma with Etodolac to get the concentration of 50, 125, 300, 550, 900, 1300, 1700, 2100 ng/mL. Quality control samples were prepared by spiking blank plasma with 50, 100, 600, 1000, 1500 ng/mL of Etodolac. The stock solutions were stored at 4-8°C.

Sample preparation

Retrieve the frozen STD Blank, STD Zero, CC, QC (also used as validation samples) samples and subject samples from the deep freezer and allow them to thaw at room temperature, vortex to mix. Remove the caps from the polypropylene tubes. Aliquot 300 µL of each sample [STD Blank, STD Zero, CC, QC (also used as validation samples) and subject samples] into pre-labelled polypropylene tubes. The internal standard (50 µL of the 2 µg/mL solution) was added to all vials except the blank samples to which 50 µL of diluent (water: MeOH 20:80 v/v) was added, vortex to mix. Process the samples using Orochem extraction cartridge, 30 mg 1CC on positive pressure solid phase extraction manifold as per the procedure given below. Standard stock solutions of Etodolac (1.2 mg/mL) and the IS(2 µg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water/methanol (20:80, v/v; diluent).

The cartridges were conditioned using 1ml of 100 % methanol. It was acidified with 1 ml of 1% formic acid. Then 550 µL sample (Analyte + IS) was added to it. It was washed twice with 1 ml of 1% formic acid. Finally it was eluted with 500µL of mobile phase (Acetonitrile: 0.5% Formic Acid) vortexes for 1 minute and 2 µL of the sample was injected.

Bio analytical method validation

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the total range 52.393ng/mL to 10530.432, including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted (1/x) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing five sets of QC samples in a batch. The between batch precision and accuracy were determined by analyzing five sets of QC samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 15% or better and the accuracy was 100_15% or better for QC samples.

Recovery of Etodolac from the extraction procedure was determined by a comparison of the peak area of Etodolac in spiked plasma samples (five each of low, medium and high QC samples) with the peak area of Etodolac in samples prepared by spiking extracted drug-free plasma samples with the same amounts of Etodolac at the step immediately prior to chromatography. Similarly, recovery of the IS was determined by comparing the mean peak areas of extracted QC samples (n¼5) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (5-8^{O}C), and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels (100 and 1500 ng/mL as low and high values) with five determinations for each.

Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use. The present method was validated in accordance with current acceptance criteria. Selectivity was assessed by analyzing blank plasma samples obtained from six different sources with six samples at LLOQ concentrations spiked using the biological matrix of any one source. Randomly selected blank human plasma sources were taken to determine the extent to which endogenous human plasma interfere with the analyte or the internal standard. No significant interference was observed in six different sources of human plasma samples. Representative chromatograms illustrating the specificity of the method are shown in Figures1 – 4. The calibration equation was determined by least squares linear regression (weighting 1/x2) over the range 50 to 2000 ng/mL in plasma.

Assay precision and accuracy (inter-day and intra-day) values were determined across three precision and accuracy batches by analyzing six replicates each of LOQQC, LQC, MQC, and HQC samples in each batch. One of the precision and accuracy batch was performed by different analyst to ensure ruggedness of the method. The stabilities were assessed under varying storage and handling conditions and determined by calculating the percentage nominal of LQC and HQC samples against freshly prepared calibration curve standards and compared with bulk spiked comparison samples (CS).

As a part of method validation freeze thaw, bench-top, short term stability, long term stability was also evaluated. Recovery of the developed method can be evaluated by analyzing six replicates of analyte along with internal standard by comparing the analytical results for extracted samples at three concentrations (equivalent to LQC, MQC and HQC) with un-extracted samples that represent 100 % recovery. The percentage recovery of analyte and internal standard (IS) were calculated using appropriate chromatographic conditions.

RESULTS AND DISCUSSION

The assay was found to be linear for Etodolac concentrations in the range 50 to 2000 ng/mL. The main working parameters for LC AND MS are presented in Tables 2&3. The precision and accuracy were studied satisfactory at four QC concentrations for Etodolac. The intraday precision and accuracy of the method at QC levels (50.00, 125, 300, 550, 900, 1300, 1700 and 2000 ng/mL, n = 6) were 0.1365, 1.4162, 2.5538, 0.1259, 0.3003, 3.2578, 1.2186 and 3.1915 and 99.7, 100.2, 101.9, 98.5, 99.2, 104.3, 99.7 and 96.5 respectively. The inter day precision and accuracy of the method at QC levels (n=6) were 1.5732, 1.8606, 3.9849, 3.9067, 0.6146, 0.4456, 4.9122 and 3.3657 and 95.7, 99.2, 101.2, 101.7, 100.6, 102.0, 100.1, 100.9 and 94.9 respectively. The results obtained from measurement of linearity, precision and accuracy, results were showed that no significant degradation was observed under the test conditions which indicate that compounds are highly stable in plasma.

The values obtained for the stability studies presented in Tables 4-9, are within the acceptance criteria. Recovery of Etodolac was evaluated by comparing mean analyte responses of six processed samples of lower limit (LLQC), low (LQC), medium-1 (MQC-1), medium-2 (MQC-2) and high (HQC) quality control samples to mean analyte responses of six appropriately diluted pure diluted solutions. Mean recovery values are 46.1428, 87.3295, 535.8317, 893.6258, and 1327.5733 % at lower limit, low, medium-1, medium-2 and high quality control levels respectively. Global mean recovery of Etodolac was 83.51 %. Mean recovery value for the internal standard was 96.10 % and it is within the limit.

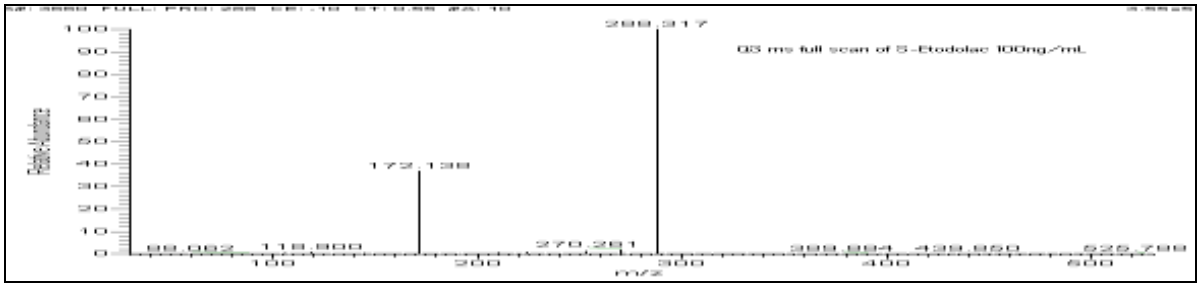


Fig 1: Etodolac MS Profile

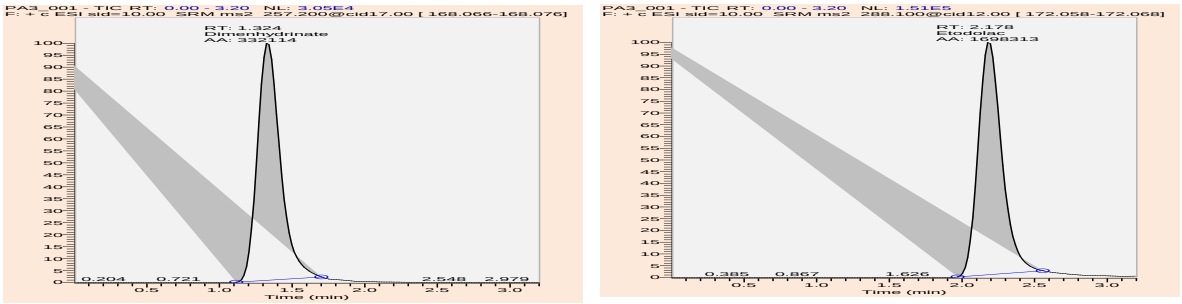


Fig 2: Etodolac and Is Representative Chromatograms from Unextracted Standard

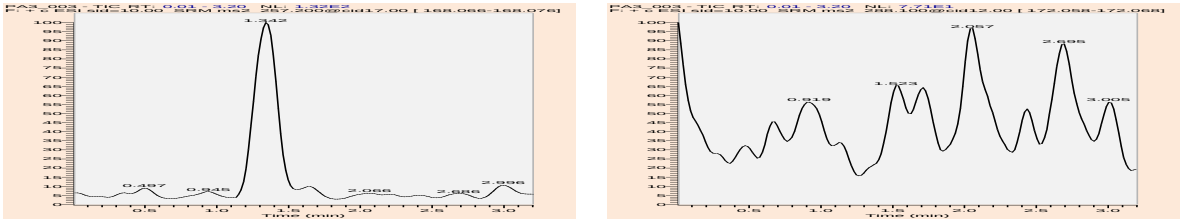


Fig 3: Etodolac and IS representative chromatograms from blank

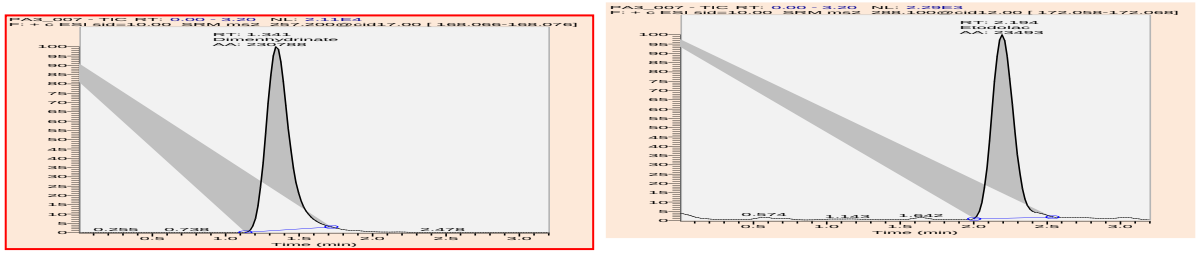


Fig 4: Etodolac and IS representative chromatograms from standard

Table 4: Global Statistics of Recovery Studies for Etodolac and Internal Standard

QC ID	Actual Conc. (ng/mL)	Mean Values				Mean	±S.D.	% CV	% Accuracy
		P&AI	P&A II	P&A III	P&A IV				
		Inter day-1		Intraday-2					
HQC	1480.962	1327.5733	1472.2078	1508.0458	1531.5287	1435.9423	91.48864	6.4	97.0
MQC 1	1007.054	893.6258	964.1603	1005.5357	1022.4887	954.4406	57.37749	6.0	94.8
MQC 2	604.232	535.8317	571.0858	581.6133	612.5892	562.8436	31.65358	5.6	93.2
LQC	102.72	87.3295	92.2662	94.4927	99.7763	91.3628	5.16301	5.7	88.9
LLOQ QC	52.387	46.1428	49.6012	49.9810	49.0583	48.5750	1.74363	3.6	92.7

Table 5: Concentrations of Etodolac in Short Term Stability Data for Stock Solution Prepared in Human Plasma

Global Statistics		
Mean Recovery %	83.50	96.10
+S.D.	9.435	2.515
%C.V.	11.3	2.6

Table 6: Concentrations of Etodolac in Long Term Stability Data for Stock Solution Prepared in Human Plasma

Duration	**0.0 hrs**	**14 hrs 53 min**	**0.0 hrs**	**14 hrs 51 min**	**0.0 hrs**	**14 hrs 46 min**
Concentration	HQC		LQC		ISTD	
(µg/mL)	1.478	1.476	0.106	0.106	2.022	2.001
Mean	132431318	136280290.7	9650191	9660397	30669484	31681629
±S.D.	3832991.53	3115206.96	220972.7	350236.5	586470.97	585512.5
%C.V.	2.9	2.3	2.3	3.6	1.9	1.8
% Stability	103		100.1		104.4	

Table 7: Concentrations of Etodolac in Bench Top Stability Data for Sample Solution Prepared in Human Plasma

Duration	0.0 hrs	08 days 15 hrs 43 min	0.0 hrs	08 days 15 hrs 43 min	0.0 hrs	08 days 15 hrs 21 min
Concentration	HQC		LQC		ISTD	
(µg/mL)	1.479	1.476	0.106	0.106	2.022	2.001
Mean	50777486.5	51102607.2	3257850	3229327	8130452.7	8130722
±S.D.	860161.34	636289.24	57382.21	144811.7	74837.55	98204.04
%C.V.	1.7	1.2	1.8	4.5	0.9	1.2
% Stability	100		94.5		101.1	

Table 8: Concentrations of Etodolac in interim stability data for sample solution prepared in human plasma

Sr. No.	At 0.0 hrs		13 hrs 42 min	
	HQC	**LQC**	**HQC**	**LQC**
	Actual concentration (ng/mL)			
	1483.684	102.908	1480.962	102.72
Mean	1534.4798	104.8868	1509.34	104.0448
±SD	45.80759	3.89892	40.84501	4.93136
%CV	3	3.7	2.7	4.7
% Stability			98.5	99.4

Table 9: Concentrations of Etodolac in Freeze and Thaw Stability Data for sample Solution Prepared in Human Plasma

Sr. No.	At 0.0 hours		07 days 08 hrs 34 min	
	HQC	**LQC**	**HQC**	**LQC**
	Actual concentration (ng/mL)			
	1483.684	102.908	1480.962	102.72
Mean	1521.1785	104.6685	1519.347	99.8303
±SD	33.47244	3.21466	23.48123	2.38878
%CV	2.2	3.1	1.5	2.4
% Stability			100.1	95.6
Sr. No.	**At 0.0 hours**		**FT Cycle IV**	
	HQC	**LQC**	**HQC**	**LQC**
	Actual concentration (ng/mL)			
	1483.684	102.908	1480.962	102.72
Mean	1521.1785	104.6685	1570.312	99.7792
±SD	33.47244	3.21466	35.98825	3.13171
%CV	2.2	3.1	2.3	3.1
% Stability			103.4	95.5

CONCLUSION

The LC-MS/MS method described for Etodolac was simple, rapid, reproducible and suitable or their determination in human plasma. This method also has a good sensitivity, specificity and is also suitable for high throughput clinical sample analysis. There were no significant interferences and matrix effects by endogenous compounds throughout the analysis. These methods can also be used as therapeutic drug monitoring technique to evaluate the pharmacokinetic parameters of drug molecules in human plasma. The developed and validated method has its own advantage and significance which can be applied for successful clinical pharmacokinetic studies.

The method was applied successfully to the analysis of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of ETD. The accuracy and precision data gives a result, which were within the acceptance limits. Consistent recoveries were observed. The method

is specific in the presence of different anticoagulants [CPDA and EDTA] and matrices collected from different sources. The desired sensitivity was achieved with an LLOQ, which has within- and between-batch coefficients of variance [CVs], respectively. Electrospray ionization (ESI) technique has proven effective in generating ions close to the protonated molecule with sufficient intensity to monitor quantitatively, accurately and selectively.

The simplicity of these methods, use of rapid extraction, chromatographic separation, mass spectrometric detection and sample turnover rate make them attractive procedures in high-throughput bioanalysis of analytes. The method was applied successfully to analysis and quantification of concentrations of analytes in plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses.

However further studies needed in the pharmaceutical field to build up more sophisticated bioanalytical assay methods for the quantification of drug[s] and/or its metabolite[s] in human biological matrix. i.e. Serum, tissue and urine.

In a "NUTSHELL" the LC-MS/MS methods described in this work are reproducible, specific and sensitive enough for the selective and reliable determination of molecule in human plasma for routine bioequivalence and pharmacokinetic analysis and were validated in the specified ranges according to internationally accepted criteria.

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