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R E S E A R C H A R T I C L E

Estimation of Levetiracetam in Human K_2EDTA Plasma in range from 0.253 µG/ML to 35.060 µG/ML by using LC-MS/MS

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A B S T R A C T

A simple, sensitive and selective method for the determination of Levetiracetam by using rapid high-performance liquid chromatography/positive electroscopy ionization tandem mass spectroscopy was developed. The method consists of precipitation extraction with Acetonitrile followed by the analysis of the extracted sample by liquid chromatography-mass spectroscopy (LC-MS/MS) in selective reaction monitoring mode using electrospray ionization mode (ESI). Chromatography was performed on a C_{18} reverse phase column, Methanol: Acetonitrile: 10mM ammonium acetate (45:45:10) as a mobile phase. The assay exhibited a linear dynamic range of 1 to 40µg/ml for Levetiracetam in human plasma. Stability assessment was also included. A run time of 3.0 min for each sample made it possible to analyse healthy volunteers participating in pharmacokinetics drug-drug interaction studies.

Keywords: LC-MS/MS, Human Plasma, Bioanalytical, Levetiracetam, Validation.

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

Levetiracetam is a novel antiepileptic drug which is structurally and mechanistically dissimilar to other antiepileptic drugs[1]. Its pharmacokinetic profile is linear with respect to dosage, its bioavailability is close to 100%,

it undergoes only insignificant hepatic metabolism to inactive metabolites, it does not induce hepatic enzymes and about 91% of the dose is excreted via the renal route[2]. Therefore, it is close to a drug with ideal pharmacokinetic properties. Nevertheless, it is recommended to monitor the plasma concentrations of levetiracetam to optimize the therapeutic effect, especially in patients with renal impairment, in the elderly where the half-life of the drug is extended[3] and in children, where the half-life is shortened[4]. There are only a few papers published reporting therapeutic drug monitoring methods of levetiracetam.

Several methods to determine Levetiracetam with HPLC or gas chromatography-mass spectrometry have been previously described[5-11]. The assays used relatively large plasma sample volumes (up to 2 mL) and either multiple-step liquid or solid-phase extraction procedures.

In this report, we describe a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method developed and validated for the quantification of Levetiracetam in human plasma and utilizing a single-step extraction and a chromatographic separation. It is essential to establish an assay capable of quantifying Levetiracetam at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Levetiracetam.

2. Experimental

Chemicals and Solvents

Levetiracetam drug substance and Rimantadine Hydrochloride (Internal Standard) was obtained from IDDS (Hyderabad, India). The chemical structures are represented in Fig.1. HPLC-grade Methanol and Acetonitrile was purchased from JT Bakers. Ammonium Acetate was purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q water system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

Table.1. Analytical Method Details

Name of the Drug	Levetiracetam
Name of the Analyte	Levetiracetam
Molecular Formula of Levetiracetam	$C_8H_{14}N_2O_2$
Molecular Weight of Levetiracetam	170.21g/mol
Name of Internal Standard	TrazodoneHCl
Molecular Formula of TrazodoneHCl	$C_{19}H_{22}ClN_5O.HCl$
Biological Matrix Used	Homogeneous K_2EDTA human plasma (MV032/PM/01/12) obtained by pooling screened K_2EDTA human plasma was used as blank for analysis and also for the preparation of calibration curve standards and quality control samples. The pooled plasma was used for dilution purpose also, during the evaluation of dilution integrity of the analytical method.

LC/MS/MS instrument and conditions:

The high-performance liquid chromatography (HPLC) SILHTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-20 AD VP binary pump, a DGU20A3 Degasser, and a SIL-HTC auto sampler equipped with a CTO-10AS VP thermo stated column. The chromatography[12-14] was on Thermo BDS Hypersil C18, (5 µm, 4.6 x 50mm) at a temperature of 20^0C. The isocratic mobile phase composition was a mixture of 45:45:10 ACN: MeOH: 10 mM Ammonium acetate, which was pumped at a flow rate of 0.8 mL/min. Mass spectrometric detection was performed on a TSQ Quantum Discovery MAX triple quadrapole instrument (Thermo Finnigan, USA) using the Multi reaction monitoring (MRM) mode. A turbo electrospray ionization (ESI) interface in positive mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on LC Quan 2.5.6. Software package (Thermo).

(a) Levetiracetam (b) Rimantadine Hydrochloride

Fig.1. Chemical structures for Levetiracetam and Rimantadine Hydrochloride

Table.2. Main working parameters of the tandem mass spectrometer

Parameters	Value
Spray voltage	3000
Sheath gas pressure	20
Auxiliary gas pressure	15
Capillary temperature	300
Tubelens offset	130 and 48 (Analyte and IS)
Skimmer offset	0(Analyte) and -12 (IS)
Collision energy	15(Analyte) and 14 (IS)
Polarity	Positive
Mode of analysis	MRM
Ion transition for Levetiracetam, m/z	171.543±0.5/ 126.134±0.5
Ion transition for Rimantadine, m/z	180.668±0.5/163.136±0.5

Sample preparation:

Standard stock solutions of Levetiracetam (40 mg/mL) and the IS (2 mg/mL) were separately prepared in Methanol. Working solutions for calibration and controls were prepared by appropriate dilution in 70:30 Methanols: water. The IS working solution (500 ng/mL) was prepared by diluting its stock solution with diluent (80:20) methanol: water. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain Levetiracetam concentration levels of 1, 2, 4,8,15,20,30 and 40 µg/mL, as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent

weighing of standard drug, at concentrations of 1.2 (LLOQ), 2.8 (Low), 15.0 (medium) and 24.7 µg/mL (high), as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in Ria Vials (Tarson, 5 mL) and stored in a freezer at below -80^{0}C until analyses. A plasma sample (0.150 mL) was pipetted into a 2-mL centrifuge tube, 500 µL of IS working solution (500 ng/mL) were added. After vortex mixing for 10 s, a 1.0-mL Acetonitrile was added and the sample was vortex-mixed for 10 s. Centrifuge the centrifuge tubes at 14000 rpm at 10^{o}C for 10 min, transfer approximately 0.8mL of supernatant to HPLC vials and a 10-µL aliquot was injected into the chromatographic system.

Bioanalytical 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 1–40 µg/mL, including the lower limit of quantitation (LLOQ). The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression[15] 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 six sets of QC samples in a batch. The between batch precision and accuracy were determined by analyzing six 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 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations. Recovery of Levetiracetam from the extraction procedure was determined by a comparison of the peak area of Levetiracetam in spiked plasma samples (six each of low, medium and high QCs) with the peak area of Levetiracetam in samples prepared by spiking extracted drug-free plasma samples with the same amounts of Levetiracetam at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples (n = 6) 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 (-80^{0}C), and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels (1 and 40 µg/mL as low and high values) with five determinations for each.

3. Results and Discussion

Mass spectrometry:

The analysis of Levetiracetam from human plasma is of major interest in pharmaceutical research. Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC/MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. The product ion mass spectra of Levetiracetam and the IS are shown in Fig. 2. Predominant ion in the Q1 spectrum was used as the precursor ion to obtain product ion spectra. The collisionally induced dissociation (CID) mass spectrum of Levetiracetam shows the formation of characteristic product ions at m/z 126.134. The CID mass spectrum of the IS shows the formation of characteristic product ions at m/z 163.136. The most sensitive mass transition was from m/z 171.543 to 126.134 for Levetiracetam and m/z 179.668 to 163.136 for the IS. LC/MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

Method development

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of ACN: MeOH: 10 mM Ammonium acetate (45:45:10) could achieve this purpose and was finally adopted as the mobile phase. The proportion of organic solvent eluted the analyte and the IS at retention times of 0.7 and 2.0 min, respectively. A flow rate of 0.8 mL/min produced good peak shapes and permitted a run time of 3.0 min. Extraction with Acetonitrile was used for the sample preparation in this work. Extraction with Acetonitrile can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC/MS/MS analyses. An Extraction with Acetonitrile was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. The average absolute recoveries of Levetiracetam from spiked plasma samples was 68.6 ± 2.0 % and the recovery of the IS was 91.3 ± 1.8% at the concentration used in the assay (500 ng/mL). Recoveries of the analytes and IS were good, and it was consistent, precise and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

Choosing the appropriate internal standard is an important aspect to achieve acceptable method performance, especially with LC/MS/MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled

internal standards for all analytes should be used, but these are not commercially available. Therefore, we opted for Rimantadine Hydrochloride commercially available. In addition its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. All validation experiments in this assay were performed with matrices obtained from different individuals. As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

Assay performance and validation:

The eight-point calibration curve was linear over the concentration range 1–40µg/mL. The calibration model was selected based on the analysis of the data by linear regression with intercepts and weighting factors (1/x, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighting factor. Linear regression equation for the calibration curve is y =m x + c here y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.9997_0.0004; Table 2 summarizes the calibration curve results. The Calibration curve obtained for Levetiracetam depicted in Fig. 3.

The selectivity of the method was examined by analyzing six blank human plasma extract, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Excellent sensitivity was observed for a 10-µL injection volume. The MRM chromatograms obtained for an extracted plasma sample are depicted in Fig. 4.

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 1 µg/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit (1 µg/mL) was ten-fold greater than the Mean response for the peak in eight blank human plasma samples at the retention time of the analyte. The between-batch precision at the LOQ QC was 2.6%, and the between-batch accuracy was 96.6% (Table 3). The within batch precision was 6.1% and the accuracy was 99.2 for Levetiracetam. The middle and upper quantification levels of Levetiracetam ranged from 2.6 to 24 µg/mL in human plasma. For the between-batch experiments the precision ranged from 2.9 to 5.4 % and the accuracy from 101.0 to 104.3 % (Table 3). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria (≤15%). The upper concentration limits can be extended with acceptable precision and accuracy by a fourfold dilution with control human plasma. These results suggest that samples with concentrations greater than the upper limit of the calibration curve can in this way be assayed to obtain acceptable data.

Stability studies:

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h).

Samples were extracted and analyzed as described above, and the results are given in Table 4. These results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the auto sampler for 48 h was also assessed. The results indicate that solutions of Levetiracetam and the IS can remain in the auto sampler for at least 48 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table 4). The data representing the stability of Levetiracetam in plasma at two QC levels over three freeze/thaw cycles are given in Table 4. These tests indicate that the analyte is stable in human plasma for three freeze/thaw cycles, when stored at below -80^0C and thawed to room temperature. Table 4 also summarizes the long-term stability data for Levetiracetam in plasma samples stored for a period of 24 days at below -80^0C. The stability study of Levetiracetam in human plasma showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of Levetiracetam in plasma samples at below -20^0C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies. The stability of the stock solutions was tested and established at room temperature for 6-8h, 72h, and under refrigeration (-4ºC) for 21 days (data not shown). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Application of the method:

The present method was applied for a randomized cross-over bioequivalence study of two different formulations in 12 healthy male volunteers. After single oral administration of the drug blood samples were collected at a suitable time intervals. This method was successfully used to measure the plasma concentrations of Levetiracetam. Various pharmacokinetic parameters established and compared for the both of the preparations.

Table.4. LOQ (Limit of Quantification) Determination

Table No 189: Results for LOQ Determination Levetiracetam		
Injection Number	Observed Concentration	
	ULOQ	LLOQ
1	32.917	0.234
2	33.141	0.234

3	32.864	0.221
4	33.491	0.234
5	33.213	0.239
6	33.622	0.235
N	6	6
Average	33.2080	0.2328
Standard Deviation	0.30299	0.00611
CV (Precision %)	0.9	2.6
Nominal Conc.	35.060	0.253
Accuracy (%)	94.7	92.0

Table.5. Effect of Potentially Interfering Drugs (PIDs) / Co Administered Drugs

Table No 193: Results for Effect of Potentially Interfering Drugs of Levetiracetam								
CC 005	STD 01	STD 02	STD 03	STD 04	STD 05	STD 6	STD 07	STD 08
Nominal Value (µg/mL)	0.253	0.504	3.592	7.815	14.718	21.849	28.749	35.060
Observed Concentration (µg/mL)	0.249	0.518	3.582	7.761	14.746	22.496	28.155	34.509
% Accuracy	98.6	102.9	99.7	99.3	100.2	103.0	97.9	98.4

Table No 188: Result for Calibration Curve Characteristics				
Batch ID	Y intercept	Slope	Correlation coefficient (r)	Regression Coefficient (r^2)
Heamolytic, Lipemic	-0.000655	0.0364	0.9998	0.9996

Table.6.

PID Drug Name	MQC ID	Nominal Conc.: 14.893 µg/mL Observed Conc.	% Accuracy
Paracetamol	025	15.232	102.3
	026	15.043	101.0
	027	15.325	102.9
	N	3	
	Mean	191.616	
	SD	11.067	
	CV (%)	5.8	
Caffeine	028	15.140	101.7
	029	15.157	101.8
	030	14.988	100.6
	N	3	
	Mean	195.623	
	SD	20.441	
	CV (%)	10.4	
Aspirin	031	15.068	101.2
	032	14.874	99.9
	033	15.603	104.8
	N	3	
	Mean	190.887	
	SD	4.132	
	CV (%)	2.2	
Ibuprofen	034	15.270	102.5
	035	15.156	101.8
	036	15.180	101.9
	N	3	
	Mean	184.414	

	SD	3.774	
	CV (%)	2.0	

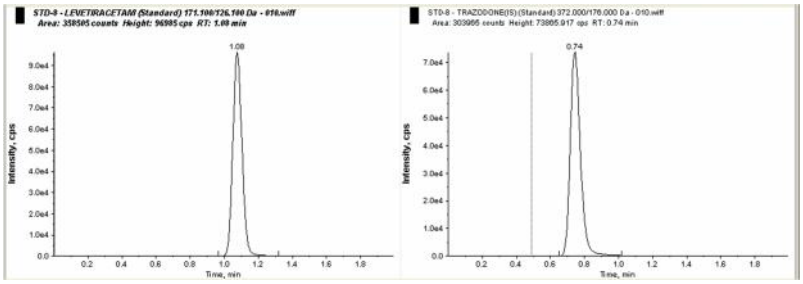

Fig.1 Representative Chromatogram for Standard-01

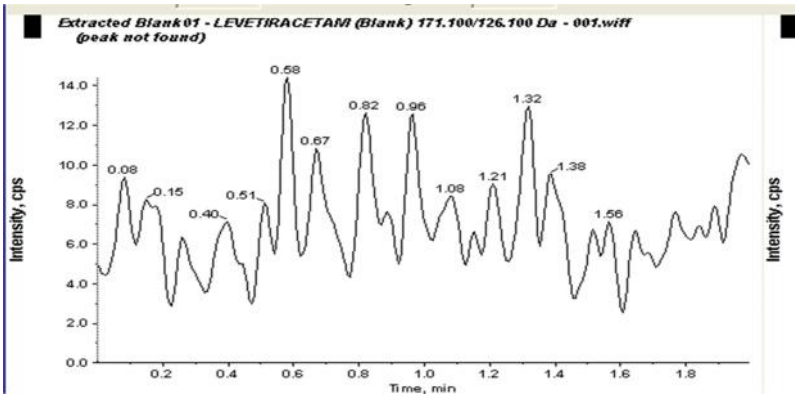

Fig.2. Representative Chromatogram for Extracted Blank

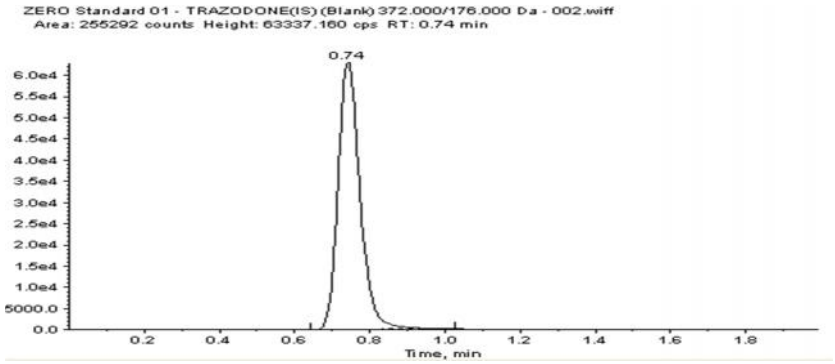

Fig.3.Representative Chromatogram for Zero Blank

4. Conclusion

In summary, a method is described for the quantification of Levetiracetam in human plasma by LC/MS/MS in negative ESI mode using multiple reaction monitoring and fully validated according to commonly accepted criteria[16,17]. The current method has shown acceptable precision and adequate sensitivity for the quantification of Levetiracetam in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The desired sensitivity of Levetiracetam was achieved with an LLOQ of 1.2µg/mL, which has within- and between-batch coefficients of variance (CVs) of 6.1% and 2.6%, respectively. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the assay and use of rapid Acetonitrile extraction and sample turnover rate of 3.0 min per sample make it an attractive procedure in high-throughput bioanalysis of Levetiracetam. The validated method allows quantification of Levetiracetam in the 1–40 µg/mL range.

5. References

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