Development and Validation of High Performance Liquid Chromatographic Method for the Determination of Levetiracetam in Human Plasma by Electospray Ionization-Mass Spectrometry

Madhira V N S Ramprasad, B. Syama Sundar*

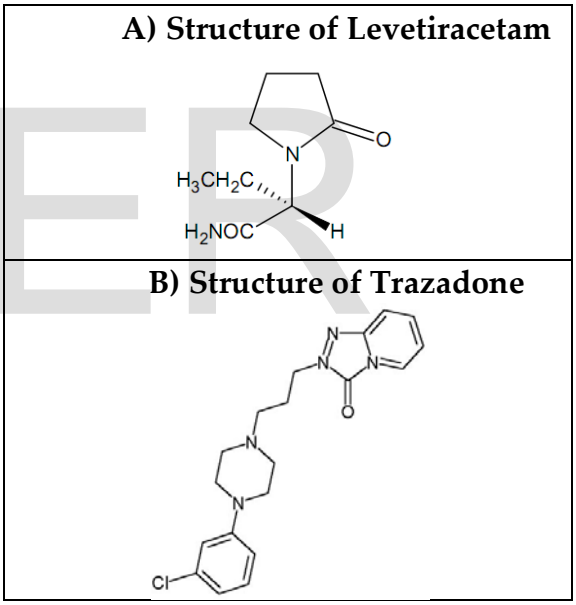
Abstract
A rapid, specific and accurate high performance liquid chromatographic method for the determination of levetiracetam in K_2EDTA human plasma using trazadone as internal standard was developed and validated using an Electrospray Ionization (ESI)-mass spectrometry detection. The extraction process involved a liquid-liquid extraction using a mixture of methyl-t-butyl ether and dichloromethane. Both levetiracetam and the internal standard were eluted under isocratic mode using a Ascentis 50 X 4.6 mm i.d, 5 µm column. The mobile phase composed a mixture of 10:90 % v/v 0.2% formic acid solution and acetonitrile at a flow rate of 1.0 mL/minute. A 50:50 splitter is used to reduce the solvent entering into the ESI. The injection volume is 5 µL. The runtime of the method is 2 minutes. The method showed good linearity in the range of 0.25 – 35.06 µg/mL. The mean recovery of levetiracetam from all the quality control samples is 45.29 % with a % coefficient of variation of of 7.5 % and recovery of internal standard was 74.28% with a % coefficient of variation of of 1.4 %. Matrix effects were not observed. The method is validated as per ICH guidelines

Keywords:, Anti-Diabetics, Electrospray Ionization , HPLC, Levetiracetam, Trazadone

1 Introduction

Levetiracetam (Keppra) is a novel antiepileptic drug recently approved by the U.S. Food and Drug Administration as a monotherapy treatment for epilepsy in case of partial seizures, or as an adjunctive therapy for partial, myoclonic and tonic-clonic seizures. Levetiracetam has potential benefits for other psychiatric and neurologic conditions such as Tourette syndrome, autism, and anxiety disorders. Chemically it is (αS)-α-ethyl-2-oxo-1-pyrrolidineacetamide (**Figure 1**) with a molecular formula of C8H14N2O2 (MW 170.20). This is a structural analog of piracetam, which binds to a synaptic vesicle protein SV2A and is believed to impede nerve conduction across synapses. The precise mechanism by which levetiracetam exerts its antiepileptic effect is still unknown [1,2].

Less than 10 percent of the drug is bound to plasma protein with steady state achieved after 48 hrs. approximately 34 % of the drug is undergoes enzymatic hydrolysis with the remaining excreted unchanged in urine. The elimination half life is 6-8 hrs [3,4]. A microdialysis study, based on using freely behaving rat model, suggests that levetiracetam is transported rapidly through the blood-brain barrier and half-life of levetiracetam in cerebrospinal fluid is much higher than in plasma [5]. Longer presence in cerebrospinal fluid enables patients to take levetiracetam only twice a day. Levetiracetam has an asymmetric carbon atom (Figure 1), and has two enantiomers, which have different pharmacokinetic properties. Isoherrane and co-workers have shown that no chiral inversion takes place in dogs [6]. A few studies deal with analysis of levetiracetam using chromatographic and electrophoresis methods [7-14]. Only one ultra-high-performance liquid chromatographic tandem mass spectrometry (UHPLC–MS–MS) method is known in the literature [15].



2 MATERIALS AND METHODS

2.1 Solvents and Chemicals

Levetiracetam (USP 99.9 % w/w) was purchased. Trazadone is kindly provided by Splendid Labs (used as Internal Standard, Purity 99.0 % w/w). HPLC grade Acetonitrile, Formic acid is purchased from Merck, Mumbai. Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

2.2 Chromatographic System & Instrument Parameters

The Chromatographic system consisted of a Shimadzu LC-10ADvp, SIL HTC Auto sampler, CTO-10ASvp Column Temperature Oven, API 3200. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using Analyst 1.5.1 software. The ESI is operated in positive ion mode Chromatographic

separations were accomplished using a Ascentis C_{18}, 5 µm, 50 mm×4.6 mm column. The mobile phase consists of a mixture of 10 parts of 0.2 % formic acid and 90 parts of Acetonitrile. The mixture was filtered through 0.22 µm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by flushing with nitrogen for 15 min. The mobile phase was pumped isocratically at a flow rate of 1.0 ml/min during analysis, at ambient temperature. The rinsing solution consists of a mixture of 90: 10 % v/v of methanol: HPLC Grade Water. The cooler of the autosampler is set 10^0C. The run time of analysis is 2.5 minutes.

2.3 Preparation of Standard Solutions

A stock solution of levetiracetam is prepared in methanol such that the final concentration is approximately 1.0 mg/mL. Stock solution of trazadone (approx 1 mg/mL) is prepared in HPLC Grade methanol. The solutions were stored at 4°C and they were stable for at least two weeks. Aqueous stock dilution of levetiracetam is prepared in diluent solution (mixture of 50: 50 % v/v of methanol: HPLC Grade water).

2.4 Sample Preparation

Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution is transferred into a 10 mL volumetric flask. The final volume is made up with screened drug-free K_2EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0.0 (Blank; no levetiracetam added), 0.252, 0.505, 3.606, 7.814, 14.726, 21.839, 28.751 and 35.063 µg/ml. Each of these standard solutions was distributed in disposable polypropylene micro centrifuge tubes (1.5 ml, eppendorf) in volume of 0.7 ml and stored at -70°C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 0.263, 0.651, 14.894 and 25.676 µg/ml respectively and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

2.5 Extraction procedure

The extraction of the plasma samples involved Liquid-Liquid Extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 500 µL is then transferred to pre-labeled polypropylene RIA Vials. 25 µL of internal standard dilution (500 ng/mL) is then added and mixed. 4 mL of the extraction solvent is then added to extract the drug and internal standard. The samples are then kept on a reciprocating shaker at 120 rpm and allowed to mix for 20 minutes. Samples are then centrifuged at 4000 rpm for 5 minutes at 4 °C. The samples are then subjected to flash-freezing using a mixture of dry-ice and acetone mixture. The supernatant is then transferred into prelabelled polypropylene tubes and allowed to evaporate to dryness under nitrogen at constant temperature of 40 °C. The dried residue is then dissolved in 200 µL of mobile phase and transferred into shell vials and containing vial inserts for analysis. 10 µL of the samples is then injected into the system for analysis. The autosampler temperature is maintained at 4 °C throughout the analysis. The column temperature oven is maintained at ambient temperature.

2.5Validation of the method

The quantitative method was validated to determine selectivity, calibration range, accuracy and precision, limit of detection (LOD), limit of quantitation, % recovery, short term, long term, freeze–thaw, and auto sampler stability. The initial assay was fully validated for levetiracetam analysis in human plasma according to FDA guidelines.

2.6 Selectivity

The selectivity of the method was evaluated by analyzing six independent drug-free K_2EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

2.7 Calibration curve

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of levetiracetam in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. Calibration curves for levetiracetam were represented by the plots of the peak-area ratio (levetiracetam / trazadone) versus the nominal concentration of the levetiracetam in calibration standards. The regression line was generated using $1/concentration^2$ factor as the mathematical model of best fit. Levetiracetam concentrations in QC samples, recovery, and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

2.7 Accuracy and precision

Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and the accuracy were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) along with three separate standard curves done in duplicates.

The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

Accuracy (%) = mean observed concentration/nominal concentration × 100

The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:

CV (%) =standard deviation/mean observed concentration× 100

2.8 Stability Studies

Autosampler, and freeze–thaw stability of levetiracetam was determined at low, medium and high QC concentrations. Bench top (9 hrs at room temperature), long term stability of the plasma matrix (11 days at −70 °C) is evaluated. To determine the impact of freeze–thaw cycles on levetiracetam concentration, samples were allowed to undergo 4 freeze (−70 °C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the levetiracetam concentrations were analyzed in triplicates and compared to the control sample that had been stored at −70 °C. Autosampler stability of extracted samples was determined by comparing levetiracetam concentration in freshly prepared samples and samples kept in autosampler at 4 °C for 48 hrs. Aqueous solutions of levetiracetam and internal standard were also evaluated for bench top stability (8 hrs at room temperature) and refrigerated stability for 2 weeks.

2.9 Recovery

Recovery was determined by comparing the area under the curve (AUC) of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of levetiracetam as in the QC samples. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 500 ng/mL.

2.10 Data analysis

Data acquisition and processing was performed by Analyst 1.5.1 software. Standard curves for quantitation of levetiracetam were constructed using a 1/concentration ^{2} weighted linear regression of the peak area ratio versus levetiracetam concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

3 RESULTS and DISCUSSION

3.1 Method Development

The ESI is optimized view to develop a sensitive and reproducible method for the determination of levetiracetam in Human Plasma. In the ESI process the molecules are subjected to a high degree of induced polarization by the application of Ion spray voltage which is usually a magnitude of 3000 – 5500 V. Depending on the polarity of the ion spray voltage the molecule undergoes protonation or deprotonation to form an ion. Conceptually, acidic molecules are electron pair acceptors and therefore ionize in the negative ion mode. Similarly basic molecules tend to ionize in positive ion mode. In the current method positive ion mode is chosen for the ion spray voltage because it resulted in higher response. A summary of the chromatographic conditions and ionization parameters is given in **Table 1**.

To facilitate the ionization process, the mobile phase is prepared such that it contains 0.2 % of formic acid. Acidic mobile phase tends to ionize the basic molecules thus resulting in better and stable response. Since both levetiracetam and internal standard are highly non-polar we employed the usage of liquid-liquid extraction process. Initial experiments were performed by using non-polar solvents like t-butyl methyl ether, dichloromethane and diethyl ether. A mixture of 70 parts of t-butyl methyl ether and 30 parts of dichloromethane gave a good recovery compared to the other methods of extraction.

Initial experiments employing conventional flow rates of 0.5-0.7 ml/min resulted in longer retention times for levetiracetam. The run time of analysis is approximately 5 minutes when a reverse phase column (50 X 4.6 mm id, C_{18}, 5 micron) is used. To increase the throughput of the analysis for the pharmacokinetic studies, we increased the flow rate to 1.0 ml/min. To facilitate a better ionization process and a faster surface evaporation of the liquid droplets, a splitter (50:50) is used. Also the organic composition of the mobile phase is kept at 90% v/v.

The column temperature is maintained at ambient. At the reported flow rate, peak shape was acceptable. There was no interference in the drug and internal standard, from the extracted blank. The use of volatile buffers for mass spectral analysis has been discussed elsewhere [16]. Acetate buffer and formate buffers were initially used for the experiment Formate buffer resulted in better ionization response. Therefore the concentration is optimized at 0.2% v/v.

Extraction methods were initially attempted using Protein precipitation technique. Precipitation technique was adopted using Acetonitrile and or Methanol. Initial experiments of protein precipitation were done using 1: 3 ratio of plasma : Organic solvents. The recovery of the levetiracetam is moderate while that of the internal standard is relatively unchanged as compared with liquid-liquid extraction.

Table 1: Optimized Chromatographic Conditions and Mass Parameters

Chromatographic conditions		Ionization Parameters	
Column Name	Ascentis (50 x 4.6) mm, 5µ	Ion spray Voltage	5000 V
Mobile Phase	Acetonitrile: Buffer Solution ::90:10, v/v	Curtain gas	35.00
Column Oven temperature	40° C	Heater temperature	500 ° C
Auto-Sampler Temperature	4° C	GS 1	40.00
Injection Volume	5 µL	GS 2	45.00
Flow Rate	1.0 ml/minute	CAD Gas	6.00
Run Time	2.5 minutes	Interface heater	ON

Drug Name	Q1 Mass	Q3 Mass	Dwell Time (msec)	Compound Parameters	Start	Stop
Leveteracetam	171.100	126.100	200	Declustering Potential (DP)	25.00	25.00
				Collision Cell Entrance Potential (CEP)	14.36	14.36
				Collision Energy	30.00	30.00
Trazadone	372.000	176.000	200	Declustering Potential (DP)	50.00	50.00
				Collision Cell Entrance Potential (CEP)	17.16	17.16
				Collision Energy	59.00	59.00

Since the noise effects in solid phase extraction (SPE) method are similar to that of liquid-liquid extraction, we have done the final analysis using liquid-liquid extraction (LLE). SPE methods although render a neat sample for final analysis, polar interferences do enter into the final sample during reconstitution. SPE is further expensive as compared to LLE technique. The recovery of levetiracetam and internal standard was high when Ethyl acetate was used individually.

3.2 Detection and chromatography

Figure 2 and **Figure 3** shows the typical mass spectra of levetiracetam and trazadone. The multiple reaction monitoring (MRM) scan for levetiracetam indicated a parent ion of 171.1 (Q1) and two product ions (154.1 and 126.0). The most stable and reproducible transition (Q1/Q3) is found to be 171.1 / 126.0 Da. The multiple reaction monitoring (MRM) scan for trazadone indicated a parent ion of 372.0 (Q1) and two product ions (176.1 and 148.1). The most stable and reproducible transition (Q1/Q3) is found to be 372.0 / 176.0 Da. The retention times for levetiracetam and trazadone were 0.88 and 1.50 min, respectively.

Figure 2: Typical MRM scan spectra for levetiracetam showing parent and product ions

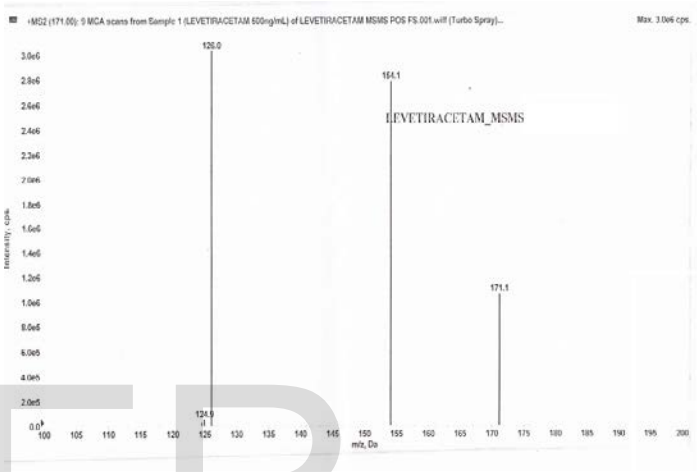
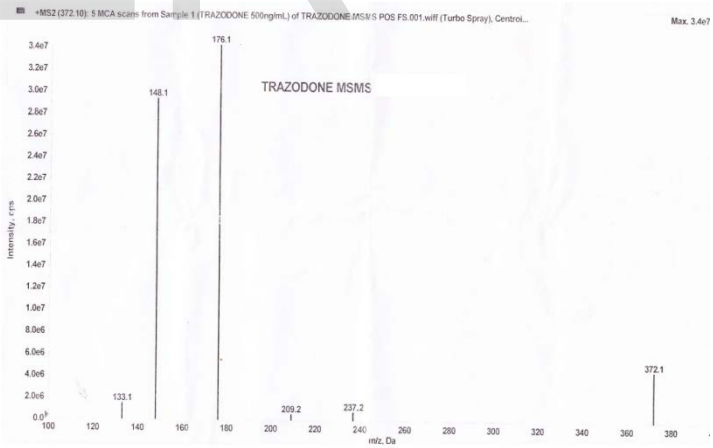


Figure 3: Typical MRM scan spectra for trazadone showing parent and product ions



4 Method validation

4.1 Calibration curves

A system suitability exercise is performed before the initiation of the validation. A system is assumed to be suitable for analysis if and only if the % CV for the retention times of levetiracetam and internal standards is less than 2 %. For preparation of pooled plasma for the validation, six different lots of blank plasma were screened for specificity. The chromatograms are represented in **Figure – 5.** All the lots were found to have no significant endogenous interferences at the retention times of the analyte and the internal standard. The same human EDTA plasma lots free of interfering substances were used to prepare the calibration curve standards and the quality control samples for the validation study.

Calibration curves for levetiracetam in human plasma were fitted by weighted 1/concentration^2 quadratic regression, with the r^2 values of >0.99 for all curves generated during the validation. The results are presented in **Table 2.** All the standards demonstrated an accuracy within ± 3% of their respective nominal concentrations. Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and r^2 value is depicted in **Figure – 4**.

Table 2: Results of regression analysis of the linearity data

Summary of Calibration Curve Characteristics For Levetiracetam				
Replicate ID	**Y intercept**	**Slope**	**Correlation Coefficient**	**Regression Coefficient**
			(r)	**(r^2)**
1	-0.000137	0.0349	0.9991	0.9982
2	-0.00104	0.0384	0.9998	0.9996
3	-0.000204	0.0369	0.9995	0.9990
N	3	3	3	3
Mean	0.000	0.037	0.999	0.999

Figure 4: Calibration Curve of levetiracetam (Curve – 1)

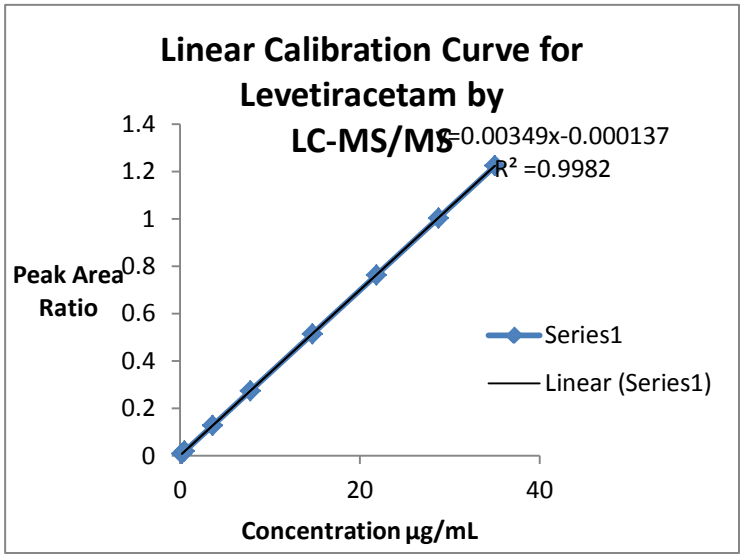
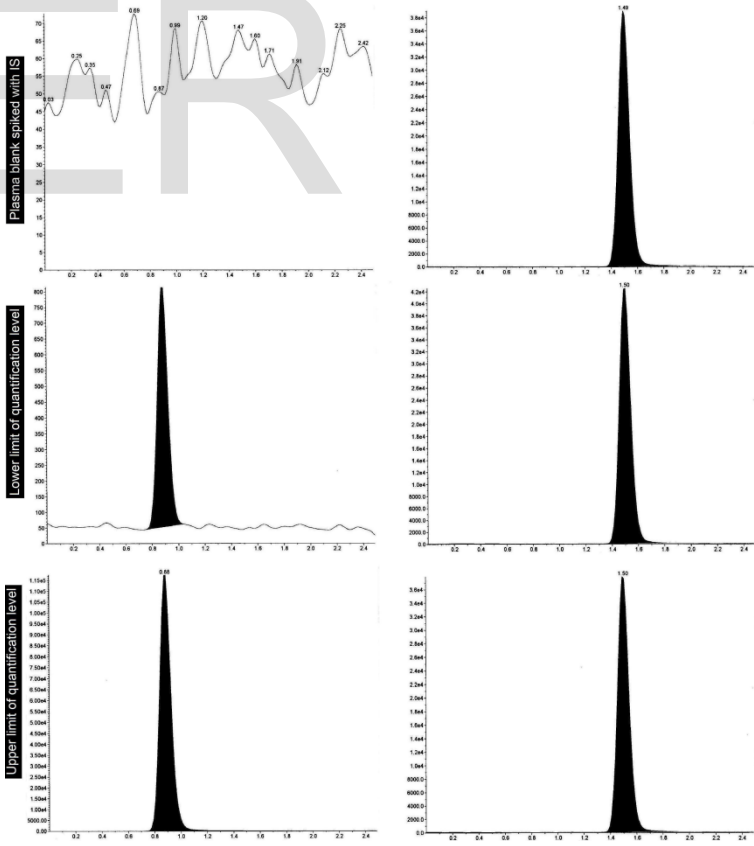


Figure-5: Representative chromatograms of Zero blank, LQC and HQC (top to bottom)



4.2 *Accuracy and precision*

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation is presented in **Table 3**. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision for all QC concentrations is within the acceptable limit recommended by the USFDA [17].

Table 3: Intra and Inter day accuracy and precision

	Nominal Concentration (µg/mL)			
	LLOQ	LQC	MQC	HQC
	0.263	0.651	14.894	25.676
Precision and Accuracy Batch – 1				
Mean	90.96	100.62	99.83	96.26
S.D.	0.01677	0.02637	0.20142	0.46108
% CV	7.0	4.0	1.4	1.9
Precision and Accuracy Batch – 2				
Mean	99.97	104.74	101.92	99.17
S.D.	0.0124	0.0222	0.4579	0.5723
% CV	4.7	3.2	3.0	2.2
Precision and Accuracy Batch – 3				
Mean	89.33	101.05	101.00	96.78
S.D.	0.0098	0.0192	0.2495	0.2722
% CV	4.1	2.9	1.7	1.1

4.3Limit of detection and limit of quantification

LOD is defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 0.263 µg/mL. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (0.263 µg/m) met the criteria for the LLOQ QC (**Table 3**). The method was found to be sensitive for the determination of levetiracetam in human plasma samples. The ULOQ has been accepted as the highest points on the standard curve with a relative standard deviation of less than 15%.

4.5 Carryover test

A critical issue with the analysis of many drugs is their tendency to get adsorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

4.6 Stability studies

The results of bench top, long term, autosampler and freeze–thaw stability are presented in **Table 4**. Determination of levetiracetam stability following three freeze–thaw cycles showed that for all QC samples there was a no significant change in the levetiracetam concentration. The stability of the aqueous solutions of levetiracetam and the internal standard were also represented in **Table-5a** and **Table 5b** respectively. Under refrigerated conditions there is no significant change in the concentration of the samples.

Table 4: Short Term, long term and Freeze Thaw stability of levetiracetam (n=6) represented as % stability

	Nominal Concentration (µg/mL)	
	0.651 (LQC)	25.676 (HQC)
Bench top stability (9 Hours)	98.6	96.3
Long-term stability (11 Days)	96.3	96.3
Freeze – Thaw stability (4 Cycles)	98.9	97.4
Autosampler stability (48 hrs)	92.4	95.1

Table 5a: Bench top and refrigerated stability of levetiracetam and trazadone in stock solution

Levetiracetam			Trazadone	
Injection No.	AQS STD 08 Response		AQS STD 08 Response	
	Fresh Stock	Room Temp. Stock Solution	Fresh Stock	Room Temp. Stock Solution
1	803201	791143	352165	374513
2	796741	789322	359348	376094
3	788497	788656	360933	370382
4	777620	796819	364009	386320
5	778155	788755	366677	383977
6	770716	788278	375587	379559
N	6	6	6	6
Average	785821.7	790495.5	363119.8	378474.2
Standard Deviation	12511.25	3259.25	7847.19	5997.57
CV (Precision %)	1.6	0.4	2.2	1.6
Stock Concentration	35.053	35.060	20.089	20.065
Correction Factor	0.9998		1.0012	
% Stability	100.6		104.4	
% Change (100 - % Stability)	-0.6		-4.4	

Table 5b: Long-term stability of levetiracetam and trazadone in stock solution

	Levetiracetam		Trazadone	
Injection No.	AQS STD 08 Response		AQS STD 08 Response	
	Fresh Stock	Refrigerator Stock Solution	Fresh Stock	Refrigerator Stock Solution
1	845648	804837	394773	396236
2	827485	801877	403928	395120
3	820977	806468	406789	398083
4	813723	796427	400318	408442
5	810796	789780	407305	403818
6	808925	794287	404911	400217
N	6	6	6	6
Average	821259.0	798946.0	403004.0	400319.3
Standard Deviation	13798.98	6510.64	4738.76	5038.59
CV (Precision %)	1.7	0.8	1.2	1.3
Stock Concentration	35.053	35.060	20.070	20.065
Correction Factor	0.9998		1.0002	
% Stability	97.3		99.4	
% Change (100 - % Stability)	2.7		0.6	

Recovery

Percentage recovery of levetiracetam was measured by dividing the peak area values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of levetiracetam at LQC, MQC and HQC levels was 41.5 %, 46.6 % and 47.8 % respectively. The overall recovery is 45.29 % with a % Coefficient of variation of 7.5 %, respectively. The recovery of internal standard is 74.28 % at MQC level concentration of levetiracetam.

5 CONCLUSION

A HPLC-ESI-MS method was developed and validated for the determination of levetiracetam in human plasma. The extraction process was a single-step liquid–liquid extraction procedure employing the use of 70: 30 methyl-t-butyl ether and dichloromethane. LLE method is usually devoid of polar interferences thus rendering the sample clean for final analysis. The noise is usually absent or at minimum as compared to precipitation or SPE techniques. There is no carryover effect. Due to the LLE method of extraction, baseline noise is minimal. Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of approximately 0.263 µg/mL, acceptable recovery, stability, specificity and excellent efficiency with a total running time of 2.0 min per sample, which is important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of levetiracetam in human subjects.

6 REFERENCES

[1] Martindale-The Complete drug reference, 2005, 34, 366.

[2] Lancelin. F, Franchon. E, Kraoul. L, Garciau. I, Brovedani. S, Tabaouti. K, Landré. E,Chassoux. F, Paubel. P, Piketty. M L, *Ther. Drug. Monit*. 2007, 29, 576-583.

[3] Patsalos, P.N. Clinical pharmacokinetics of Levetiracetam. Clinical Pharmacokinetics 2004, 43, 707–724.

[4] Radtke, R.A. Pharmacokinetics of Levetiracetam. Epilesia 2001, 45, 24–27.

[5] Tong, X.; Patsalos, P.N. A microdialysis study of the novel antiepileptic drug Levetiracetam: Extracellular pharmacokinetics and effect on taurine in rat brain. British Journal of Pharmacology 2001, 133, 867–874.

[6] Isoherranen, N.; Yagen, B.; Soback, S.; Roeder, M.; Schurig, V.; Bialer, M. Pharmacokinetics of Levetiracetam and its enantiomer (R)-a-ethyl-2-oxo-pyrrolidine acetamide in dogs. Epilepsia 2001, 42, 825–830.

[7] Contin, M.; Mohamed, S.; Albani, F.; Riva, R.; Baruzzi, A. Simple and validated HPLC-UV analysis of

Levetiracetam in deproteinized plasma of patients with epilepsy. Journal of Chromatography, B: Biomedical Sciences and Applications 2008, 873, 129–132.

[8] Martens-Lobenhoffer, J.; Bode-Bo¨ ger, S.M. Determination of Levetiracetam in human plasma with minimal sample pretreatment. Journal of Chromatography B 2005, 819, 197–200.

[9] Pucci, V.; Bugamelli, F.; Mandrioli, R.; Ferranti, A.; Kenndler, E.; Raggi, M.A. High-performance liquid chromatographic determination of Levetiracetam in human plasma: Comparison of different sample clean-up procedures. Biomedical Chromatography 2004, 18, 37–44.

[10] Shihabi, Z.K.; Oles, K.; Hinsdale, M. ; Analysis of antiepileptic drug Keppra by capillary electrophoresis. Journal of Chromatography A 2003, 1004, 9–12.

[11] Matar, K.M. Quantification of Levetiracetam in human plasma by liquid chromatography-tandem mass spectrometry: Application to therapeutic drug monitoring. Journal of Pharmaceutical and Biomedical Analysis 2008, 48, 822–828.

[12] Guo, T.; Oswald, L.M.; Mendu, D.R.; Soldin, S.J. Determination of Levetiracetam in human plasma/serum/saliva by liquid chromatography-electrospray tandem mass spectrometry. Clinica Chimica Acta 2007, 375, 115–118.

[13] Rao, B.M.; Ravi, R.; Reddy, B.S.S.; Sivakumar, S.; Chand, I.G.; Kumar, K.P., etc. A validated chiral LC method for the enantioselective analysis of Levetiracetam and its enantiomer R-a-ethyl-2-oxo-pyrrolidine acetamide on amylase-based stationary phase. Journal of Pharmaceutical and Biomedical Analysis 2004, 35, 1017–1026.

[14] Isoherranen, N.; Roeder, M.; Soback, S.; Yagen, B.; Schurig, V.; Bialer, M. Enantioselective analysis of Levetiracetam and its enantiomer R-a-ethyl-oxo-pyrrolidine acetamide using gas chromatography and ion trap mass spectrometric detection. Journal of Chromatography B: Biomedical Sciences and Applications 2000, 745, 325–332.

[15] Blonk, M.I.; van der Nagel, B.C.; Smit, L.S.; Mathot, R.A.A. Quantification of Levetiracetam in plasma of neonates by ultra performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography B 2010, 878, 675–681.

[16] Snyder AP. 1995. Electrospray: a popular ionization technique for mass spectrometry. In: Snyder AP, editor. Biochemical & biotechnological applicatons of ESI MS. Washington, DC: Americana Chemical Society 619:1-20.

[17] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration Center for Drug Evaluation and Research, 2001.

AUTHORS:

-)Madhira V N S Ramprasad,Department of chemistyry, Acharya Nagarjuna Nagar, Guntur, Andhra Pradesh 522510,E-mail:rampms11@gmail.com.
- Prof B Syama sundar, Vice-Chancellor**,** Yogi vemana university, Vemanapuram,Kadapa,Andhra Pradesh,India,Pin Code: 516003. Mail id: profbsyamsundar@yahoo.co.in