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VALIDATED LIQUID CHROMATOGRAPHIC ULTRA VIOLET METHOD FOR THE QUANTIZATION OF LEVETIRACETAM IN HUMAN PLASMA USING LIQUID-LIQUID EXTRACTION

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Abstract:

A simple, sensitive and rapid high performance liquid chromatography method with UV detection (215nm) was developed and validated for the quantification of Levetiracetam in human plasma. Following a single-step liquid-liquid extraction with diethyl ether/dichloromethane (70/30 v/v), the analyte and internal standard (Zonisamide) were separated using an isocratic mobile phase, mixture of buffer (5mM Di-Potassium hydrogen phosphate anhydrous, pH7.2): Methanol (85:15 v/v) on reverse phase C-8 Kromasil column. The lower limit of quantization was 1µg/mL, with a relative standard deviation of less than 20%. A linear range of 1µg/mL to 40µg/mL was established. This HPLC method was validated with between and within-batch precision of 5.6–8.9% and 3.9-5.3%, respectively. The between and within batch accuracy was 99.9-106.3% and 96.2-102.0%, respectively. Frequently co-administered drugs did not interfere with the described methodology. Stability of Levetiracetam in plasma was >90%, with no evidence of degradation during sample processing (autosampler) and 60 days storage in a freezer. This validated method is sensitive and simple with between-batch precision of <10% and was used in pharmacokinetic studies.

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INTRODUCTION:

Levetiracetam (Keppra) is a novel antiepileptic drug recently approved by the U.S. Food and Drug Administration as an add-on therapy in the treatment of partial onset seizures in patients. It is structurally and mechanistically dissimilar to other antiepileptic drugs¹. 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 route².

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 extended³ and in children, where the half-life is shortened^{4,5}.

Till date, no simple HPLC method has been reported for levetiracetam quantitation in plasma there are only a few papers published reporting therapeutic drug monitoring methods of levetiracetam. Two methods were using GC with NPD-detection⁶⁻⁸. Microemulsion electrokinetic chromatography with UV-detection was utilized in one method⁹, but it lacks suitable sensitivity.

Two methods facilitating chiral separation of the S- and R enantiomers of levetiracetam, one utilizing GC-MS

and the other HPLC-UV, were published recently^{10,11}. These methods were designed to investigate in dogs the pharmacokinetic and pharmacodynamic properties of the two enantiomers separately. For routine therapeutic drug monitoring in men, these methods were not appropriate.

All published HPLC-methods used reversed phase stationary phases. Because of the very polar nature of levetiracetam, a reasonable retention was only achieved with very low fractions of organic modifiers in the mobile phase. A sensitive analytical method is necessary for quantitation of the concentrations of levetiracetam in human plasma in order to support pharmacokinetic and bioequivalence studies. We here present a method for the routine quantification of the novel antiepileptic drug levetiracetam in human plasma by HPLC-UV. The procedure is very easy, quick, inexpensive and rugged.

MATERIALS AND METHODS:

Chemicals: Levetiracetam reference standards were obtained from LGC Promochem India Pvt. Ltd., Mumbai, India, and Zonisamide (internal standard) was obtained from the Torent Research Center; Ahmedabad. Chemical structures are presented in Fig. 1. Drug free plasma containing heparin as an anticoagulant was collected from Micro Therapeutic hydrogen phosphate anhydrous (GR Grade) from Merck (India). a Milli-Q system (Millipore, Bedford, MA, USA) was used.

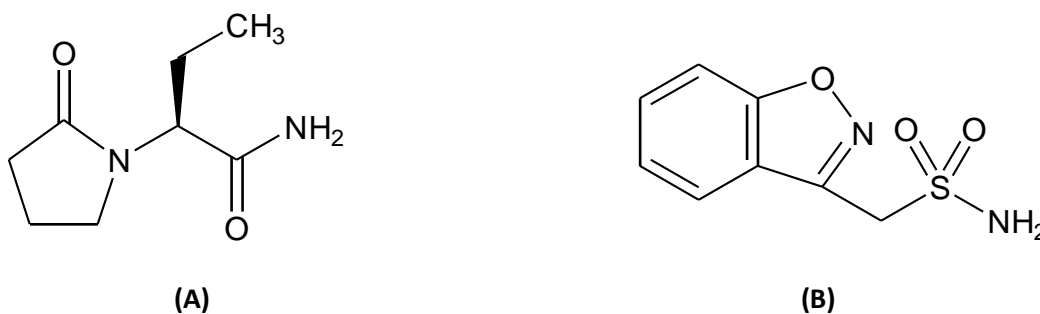


Figure 1: Chemical structures for (A) Levetiracetam, (B) Zonisamide (IS)

Chromatography: The integrated high performance liquid chromatography system (LC 2010C, Shimadzu Corporation, Kyoto, Japan) was equipped with a quaternary pump, a degasser, an auto sampler, an injector with a 100 μ L loop, a column oven, a UV detector and a data system (LC solution software version 1.21). The separation of compounds was made on a C-8 Kromasil column (150 X 4.6 mm, 5 μ) 40 °C temperature. The mobile phase was a mixture of buffer (5mM Di-Potassium hydrogen phosphate anhydrous, pH-7.2): Methanol (85:15 v/v) pumped at a flow-rate of 1.0 mL/min. Detection was set at a wavelength of 215 nm. Peak quantitation was done by peak area ratio method. All regressions and figures presented in this study were generated by Shimadzu LC solution version 1.21software.

Sample processing: A 250 μ L volume of plasma was transferred to a 4mL RIA vial, and then 50 μ L of IS working solution (50 μ g/mL) was spiked. After vortexing for 30 s, add 100 μ L of 0.025 M disodium hydrogen phosphate solution in glass test tube. Then 2.5-mL aliquot of the extraction solvent, diethyl ether/dichloromethane (7/3), was

added using handistep (Brand Eppendorf, Germany). The sample was vortex-mixed for 10 min using a Multi-Pulse Vortexer (Heidolph, Germany). The sample was then Centrifuge at rcf 1891 ± 100 for 5 minutes at 10°C using Multifuge 3S-R (Kendro Lab, heraeus, Germany). The organic layer (2.0 mL) was quantitatively transferred to a 4mL glass tube and evaporated to dryness using a TurboVap LV Evaporator (Speedoap, India) at 40 °C under a stream of nitrogen. Then, the dried extract was reconstituted in 250 μ L of Mobile phase and a 20- μ L aliquot was injected into the chromatographic system.

Bio-analytical method validation:

Calibration and control samples:

Standard stock solution of Levetiracetam (5 mg / mL) was prepared in Milli Q water and the Zonisamide ISTD (1 mg / mL) was separately prepared in methanol. Spiking solutions for calibrations curve and quality controls were prepared by appropriate dilution in Milli Q water. The IS working solution (40 μ g/mL) was prepared by diluting its stock solution with methanol: water (50:50). Spiking solutions (0.2 mL) were added to drug-

free human plasma (9.8 mL) as a bulk, to obtain Levetiracetam spiking concentration levels of 1-40 µg/mL. The quality control pools were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at -70 °C until analysis. Each validation run consisted of a double control, system suitability sample, blank samples (a plasma sample processed without an IS), a zero sample (a plasma processed with IS), calibration curve consisting of eight non-zero samples covering the total range (1µg/mL to 40µg/mL) and QC samples at three concentrations (n = 6, at each concentration).

Such validation runs were generated on six consecutive days. Calibration samples were analyzed from low to high at the beginning of each validation run and other samples were distributed randomly through the run. Linearity was assessed by a weighted (1/x²) least squares regression analysis. The calibration curve had to have a correlation coefficient (r²) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except .LLOQ, which was set at 20%. At least 67% of non-zero standards should meet the above criteria, including acceptable LLOQ and upper limit of quantization.

Specificity: Randomly selected six blank human plasma samples, which were collected under controlled conditions, were carried through the extraction procedure and chromatograph to determine the

extent to which endogenous plasma components may contribute to interference with the analyte or the internal standard. The results were compared with LLOQ (1 µg/mL).

Recovery: Recovery of Levetiracetam was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples to mean peak areas of six unprocessed reference solutions. Recovery of IS was evaluated by comparing the mean peak areas of low, medium and high quality extracted quality control samples to mean peak areas of ten unprocessed reference solutions of the same concentration.

Accuracy and Precision: Within-batch accuracy and precision evaluations were performed by repeated analysis of Levetiracetam in human plasma. The run consisted of a calibration curve plus six replicates of each LLOQ, low, medium and high quality control samples. Between-batch accuracy and precision were assessed by analysis of samples consisting of a calibration curve and six replicates of LLOQ, low, medium and high quality control samples for Levetiracetam on three separate occasions. During routine analysis, each analytical run included a set of calibration samples, a set of QC samples in duplicate and plasma samples to be determined. The overall precision of the method expressed as relative standard deviation and accuracy of the method.

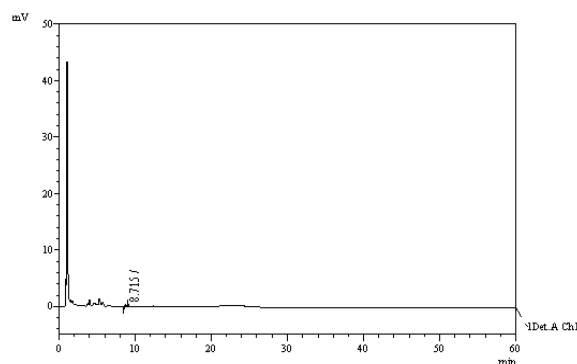
Stability: Twenty-four hour stability was examined by keeping replicates of the low and high plasma quality control

samples at room temperature for approximately 24 h. Freeze–thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 h and refrozen for 12–24 h for each cycle. Autosampler stability of Levetiracetam was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the autosampler tray for 120 h.

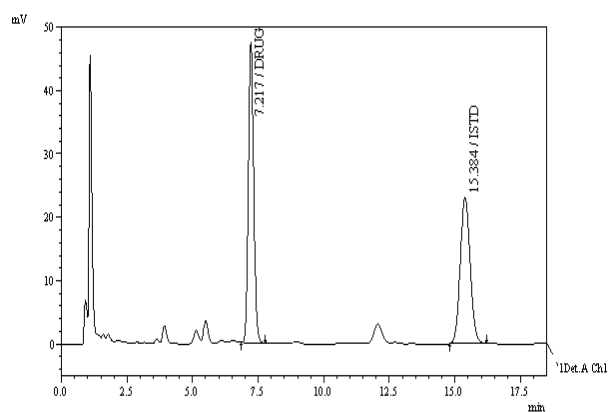
Stability of Levetiracetam in human plasma was tested after storage at approximately -70°C for 60 days. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of Levetiracetam after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

RESULTS AND DISCUSSION:

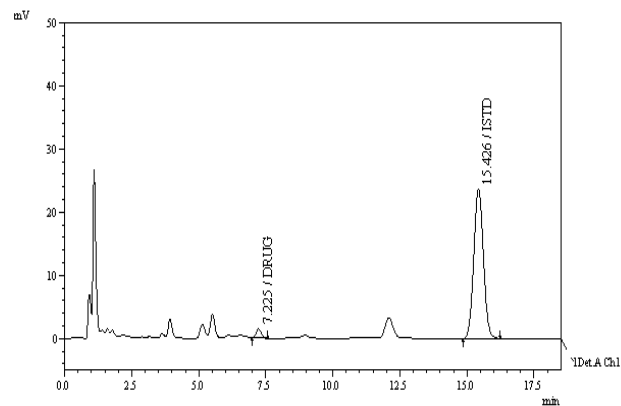
Separation: Fig. 2 shows the representative chromatograms of blank plasma, plasma samples spiked with Levetiracetam at $35\ \mu\text{g}/\text{mL}$ and at LLOQ ($1\ \mu\text{g}/\text{mL}$). The analytes were well separated from coextracted material under the described chromatographic conditions at retention times of 7.2 and 15.4 min, respectively. The peaks were of good shape, completely resolved one from another at therapeutic concentrations of Levetiracetam. No interference with constituents from the plasma matrix was observed.



(A)



(B)



(C)

Fig. 2: Chromatograms of (A) blank human plasma; (B) human plasma sample spiked with $35\ \mu\text{g}/\text{mL}$ of Levetiracetam and IS (HQC); (C) spiked human plasma sample at LLOQ ($1\ \mu\text{g}/\text{mL}$). Approximate retention times: Levetiracetam = 7.2 min; IS = 15.4 min.

Linearity and sensitivity of the assay:

The peak area ratio of Levetiracetam to IS in human plasma was linear with respect to the analyte concentration over the range 1 to 40 µg/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors (1/x, 1/x² and 1/√x). The residuals improved by weighted (1/x²) least-squares linear regression. The best fit for the calibration curve could be achieved with the linear equation $y = mx + c$ with a 1/x² weighing factor. The correlation coefficient (r) for Levetiracetam was above 0.999 over the concentration range used. Table 2 summarizes the calibration curve results for the analyte. These calibration curves were suitable for generation of acceptable data for the concentrations of the analyte in the samples during between- and within-batch validations.

The lower limit of quantification (LLOQ), the lowest concentration in the standard curve, which can be measured with acceptable accuracy and precision for the analyte from normal human plasma, was established as 1 µg/mL. The mean response for the analyte peak at the assay sensitivity limit (1µg/mL) was ≈ 8.12-fold greater than the mean response for the peak in six blank human plasma samples at the retention time of the analyte.

Extraction: In the previous studies, one of the extraction method available is protein precipitation, this method is cheap but it not clean the sample

properly due to that column back pressure increases, reduce the life of column and many problems come during the in routine clinical sample analysis. We were looking for alternative simple methods. The extraction method should also be suitable for an internal standard that is commercially available without being a stable isotope or a structural isomer of Levetiracetam. In order to develop a single step liquid–liquid extraction procedure with sufficient recovery, we investigated a large range of extraction solvents.

The absolute recovery of Levetiracetam after single extraction from plasma using chloroform, ethyl acetate and hexane were all <50%; however, when the diethyl ether/dichloromethane (7/3) was used, the absolute recovery was quite high (>75% for both Levetiracetam and IS).The commercially available substance Zonisamide was chosen as the IS for several reasons. First, Zonisamide physicochemical properties are very well in match with Levetiracetam with regard to this bioanalytical method. Second, the chromatographic and extraction properties are similar to Levetiracetam. The extraction recovery of Levetiracetam at low, medium and high quality control samples was 76.1±2.1%, 75.6±2.3% and 76.6±2.4%, respectively. It indicates that extraction recovery of Levetiracetam is independent of concentration. The recovery of IS was 75.9% at the concentration used in the assay (50µg/mL).

Table 1: Precision and accuracy of the method for determining Levetiracetam concentrations in human plasma

Conc. added ($\mu\text{g/mL}$)	Within-batch (n=6)			Between-batch (n=6)		
	Conc. found ($\mu\text{g/mL}$) (Mean \pm SD)	Precision (%)	Accuracy (%)	Conc. found ($\mu\text{g/mL}$) (Mean \pm SD)	Precision (%)	Accuracy (%)
1.050	1.0250 \pm 0.05402	5.3	97.6	1.0485 \pm 0.09335	8.9	99.9
3.000	2.9815 \pm 0.12974	4.4	99.4	3.0574 \pm 0.17216	5.6	101.9
20.000	19.2320 \pm 0.98349	5.1	96.2	20.8591 \pm 1.25533	6.0	104.3
35.000	35.6995 \pm 1.39502	3.9	102.0	37.1918 \pm 2.65166	7.1	106.3

Specificity: There were no interfering peaks present in six different randomly selected samples of drug free human plasma used for analysis at the retention times of either analyte or IS. There was no interference of Levetiracetam and IS analysis by other potentially co-administered drugs such as paracetamol, ibuprofen, aspirin, ampicillin, amoxicillin.

Accuracy of the assay: The accuracy values for between- and within-batch studies at the LLOQ and at low, medium and high concentrations of Levetiracetam in plasma were within acceptable limits (n=6) (Table 1).

Precision of the methods:

Within-batch variability of the assay: The results shown in Table 1 indicate that the assay method is reproducible for replicate analysis of Levetiracetam in human plasma within the same day.

Between-batch variability of the assay: The results shown in Table 1

indicate that the assay method is reproducible on different days.

Stability: Stock solutions of Levetiracetam (1 mg/mL) and IS (1 mg/mL) were prepared separately in methanol. The weight of analyte was corrected for purity. The solutions were stable for at least 1 month when stored at 4 °C. The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. These were performed as described in Section 2.4.5. All stability results are summarized in Table 2. Three freeze-thaw cycles and 24 h room temperature storage for low and high quality controls samples indicated that Levetiracetam was stable in human plasma under these conditions. QC samples were stable for at least 60 days if stored frozen at approximately -70 °C. Testing of autosampler stability of quality control samples (Table 2) indicated that Levetiracetam is stable when kept in the autosampler for up to 120 h.

Table 2: Stability of Levetiracetam in human plasma

Sample Concentration ($\mu\text{g/mL}$)	Concentration found ($\mu\text{g/mL}$)	% Change
Autosampler stability for 120 h		
3.000	3.2693	9.0
35.000	36.0349	3.0
Three freeze and thaw cycle		
3.000	2.8340	-5.5
35.000	34.7048	-0.8
Long term stability of analyte in matrix for 60 days		
3.000	3.0648	2.2
20.000	20.6742	3.4
35.000	35.9457	2.7
Bench top stability for 24 h		
3.000	2.9673	-1.1
35.000	36.0410	3.0

Dilution integrity: The dilution integrity was also conducted to assess whether the upper concentration limit ($40\mu\text{g/mL}$) can be extended. Quality control samples (in six replicates) at concentration $120\mu\text{g/mL}$ were diluted by four times with blank plasma, and the assay precision and accuracy were determined in a similar manner as described in Section 2.4.4. For Levetiracetam, the concentration found was $121\pm 3.7\% \mu\text{g/mL}$ and accuracy was 3.2%. The results suggested that samples whose concentrations were greater than the upper limit of the standard curve could be re-analyzed by appropriate dilution.

CONCLUSION:

The developed HPLC/UV method employing liquid-liquid extraction for sample preparation is very simple and convenient for the quantitation of Levetiracetam in human plasma

samples. The previously reported methods for the analysis of Levetiracetam in biological fluids¹⁰⁻¹³ were not too satisfactory because all of them were too expensive. The validation data also demonstrate good precision, accuracy and high extraction efficiency. The validated method allows quantification of Levetiracetam in the $1.0-40\mu\text{g/mL}$. In conclusion this paper describes a very simple and sensitive HPLC method for the quantization of Levetiracetam suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans.

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