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Novel contribution in online sample cleanup for quantitative determination of Levetiracetam in human plasma by LC-Tandem mass spectrometry

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ABSTRACT

The aim of this work was to develop and validate a simple, sensitive and rapid method for the quantitation of levetiracetam (LEV) in plasma using LC-Tandem mass spectrometry. Plasma samples were prepared by simple protein precipitation using acetonitrile; atenolol was used as internal standard (IS). Chromatographic separation was done on Luna 5 μm C18(2) (Phenomenex) 50 × 2.0 mm using gradient flow using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile: water, 95:5, *v:v*). The positive-ion mass spectrometric detection method utilized electrospray ionization and the multiple reaction monitoring (MRM) mode. The MRM ion transitions were 170.9 → 140.8 and 267.3 → 145.1 for levetiracetam and atenolol, respectively. The retention time of levetiracetam and atenolol is 3.24 and 2.96 min, respectively. The total run time was 5 min. The assay was validated over a concentration range from 1 to 100 μg/mL. The method was robust (minimal matrix effect), sensitive (LOQ, 1 μg/mL) metabolites and reproducible (The precision and accuracy for both intra- and inter-day were acceptable <15%). The method can be done on traditional LC-MS equipment. The method was effectively applied to single case study receiving toxic dose of LEV.

1. Introduction

Epilepsy is a complicated disorder characterized by two or more unexplained seizures. Epilepsy is classified according to the origin of seizure into partial and generalized seizures [1,2]. The strategy of treatment of the epilepsy varies according to seizure type and the epileptic syndrome [3]. First generation antiepileptic drugs as phenytoin, carbamazepine and sodium valproate are widely used, however because of their high risk of adverse reactions and drug interactions; they require therapeutic monitoring [4]. On the other hand second generation drugs are preferred due to favourable side effect profile and less chance of drug interactions [5].

Levetiracetam (LEV) is a brand new antiepileptic drug Figure 1. It is widely distributed since 2000. It is belongs to second generation antiepileptic drugs. Levetiracetam effect-tively used for other psychiatric and neurologic symptoms such as Tourette syndrome, autism, as well as anxiety disorders. At present, little is known concerning the mecha-nism of antiepileptic action [6]. Numerous chromatographic methods have been described for the quantification of LEV in biological fluids. Most of these methods are utilized different chromatographic techniques such as Gas chromatography (GC) with nitrogen-phosphorus detection, High performance liquid

chromatography (HPLC) and GC-MS [7,8]. There are numerous HPLC techniques [9-16], however there are few reported methods for the analysis of LEV using LC-MS/MS [17-20]. Two of which utilize a tedious solid phase extraction (SPE) procedure of sample extraction [17,19].
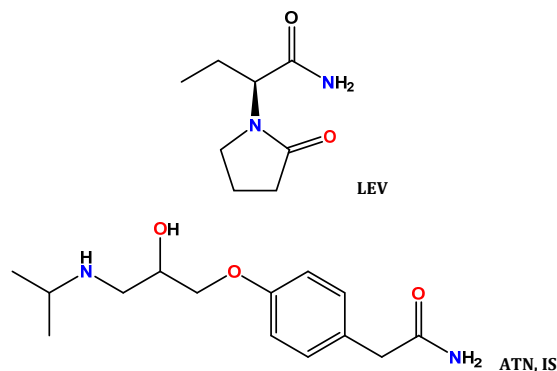

Figure 1. Chemical structure of levetiracetam (LEV) and internal standard, atenolol (ATN, IS).

The objective of the present report was to develop and validate a suitable LC-Tandem mass method for moderate throughput as in clinical or commercial reference laboratories for therapeutic drug monitoring of LEV.

2. Experimental

2.1. Materials and reagents

Levetiracetam and atenolol were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). All solvents were HPLC grade and were obtained from Merck (Kilsyth, VIC, Australia). Blank plasma was obtained from Australian Red Cross.

2.2. Equipment

The HPLC apparatus consisted of two Shimadzu LC-10AD pumps, auto sampler Shimadzu SIL-20AC-HT and a Shimadzu SLC-10A VP system controller. Detection was performed using a MDS Sciex API2000 triple quadrupole mass spectrometer (Applied Bio systems, Foster City, CA) that was operated in positive ion mode with turbo electrospray ionization sources (ESI) (Applied Biosystems Inc., Foster City, CA, USA).

2.3. Chromatographic and mass spectrometric conditions

The online sample cleanup was achieved on a Security Guard column (C18, 4 × 3 mm, 5 μm, Phenomenex, Torrance, CA, USA). The chromatographic separation was performed on a Luna analytical column (C18(2), 50 × 2.0 mm, 5 μm, Phenomenex, Torrance, CA, USA).

Three pumps were used with a 10-place switching valve to perform the online sample cleanup procedure. Mobile phase A (in pumps A & C) was 0.1% formic acid in water and mobile phase B (in pump B) was 0.1% formic acid in acetonitrile: water (95:5, *v:v*). The aqueous mobile phase A was used at 1 mL/min to load and wash the sample on the guard cartridge for 1.47 min and then a gradient elution was used with mobile phase consisting of 0.1% formic in water (solvent A) and acetonitrile: water in the ratio of 95:5 (*v:v*) (solvent B). The elution gradient was as follows: started from 10% B and kept for 1 min then raised the precent of B to 70% within 1 min, maintained at 70%B for another 1.5min and retained back to 10%B at 3.51 min. The total run time was 5 min with a total flow rate of 0.2 mL/min. The detection was made with electrospray ionization operating at positive ion mode and the tandem spectrometer was operated in the multiple reaction monitoring (MRM) mode. The mass spectrometric conditions were optimized for LEV and internal standard (IS) atenolol by continuous infusion of the standard solutions (0.03 μg/min) using a Harvard infusion pump. The turbo ion spray temperature was maintained at 400 °C and the ion spray voltage was set at 5000 V. The nebuliser gas (GS1) and the turbo gas (GS2) was 70 psi. The curtain gas (CUR) and collision gas (CAD) were both set at 40 psi. Declustering potential (DP) was 20 V for LEV and 38 V for IS. Collision energy was 27 V for LEV and 40 V for IS. The MRM transition was 170.9 → 140.8 for LEV and 267.3 → 145.1 for IS.

2.4. Preparation of stock and working solution

Stock solution of LEV was prepared by dissolving 5.00 mg of LEV in 10 mL of water, while stock solution of IS was prepared by dissolving 5.00 mg in least amount of MeOH and make up to 10mL with water. The solutions were stored at 4 °C. Standard working solutions at various concentrations were prepared freshly by diluting of appropriate aliquots of stock solution with water. Working IS solution was prepared by diluting stock solution to obtain 500 ug/mL final concentration.

2.5. Preparation of calibration standards and quality control (QC) samples

The calibration standards in human plasma were prepared by spiking 50 μL of working standard solution into 50 μL of blank plasma to achieve the concentration range of 1-100 μg/mL. QC samples were prepared at three concentration levels (5.0, 37.5 and 75.0 μg/mL) by spiking the appropriate working standard solutions into human blank plasma (50 μL).

2.6. Sample preparation

Sample preparation was performed by protein precipitation with acetonitrile. An aliquot of 50 μL plasma sample QC samples, or clinical plasma samples (blank plasma in case of standard) was mixed with 50 μL of IS working solution and 100 μL of acetonitrile. After vortex-mixing for 10 s and centrifuging at 1000 × *g* for 10 min, the supernatant was transferred into HPLC vial and 10 μL was injected to the instrument.

2.7. Method validation

2.7.1. Selectivity and matrix effect

The selectivity of the method was evaluated for potential endogenous interferences for LEV by analysing six different batches of human blank plasma. A peak or response at the respective retention times for LEV and IS with a signal to noise ratio (S/N) of less than 3:1 was considered to be insignificant. Matrix effect was evaluated by comparing peak area of LEV in spike-after preparation samples with that in neat solution. Two difference concentrations were evaluated with five samples at each level.

#### 2.7.2. Linearity and lower limit of quantification (LLOQ)

Nine non-zero calibration standards at concentrations of 1.00, 1.56, 3.12, 6.25, 12.50, 25.00, 50.00, 80.00, 100.00 μg/mL were prepared freshly for each run and analysed in three separate runs. Calibration curves were obtained by linear regression of the peak area ratios of LEV to IS against the corresponding concentrations using a weighting factor of $1/\chi^2$ (reversed square of the concentration). The LLOQ was defined as the lowest concentration in the standard curve with a precision of less than 20% and accuracy 80-120%. Deviations from the nominal concentrations should be within ±15% for the other concentrations.

2.7.3. Accuracy and precision

The intra-day accuracy and precision was determined by analysing QCs at three concentration levels using a freshly prepared calibration curve on the given day. Additional QC samples were also analysed on five different days in order to assess inter-day accuracy and precision. Precision was represented as percentage of relative standard deviations (RSD%) while accuracy was expressed as the mean of the measured concentrations as a percentage of the nominal concentration.

2.7.4. Stability test

The post-preparative stability in the autosampler at 4 °C was determined by injecting preparations of processed samples for up to 24h after the initial injection. Short-term stability in plasma (3h bench storage) was determined at ambient temperature (24±3 °C) at concentrations of QC samples. The stability was also tested after three freeze/thaw cycles using the concentrations of QC samples.
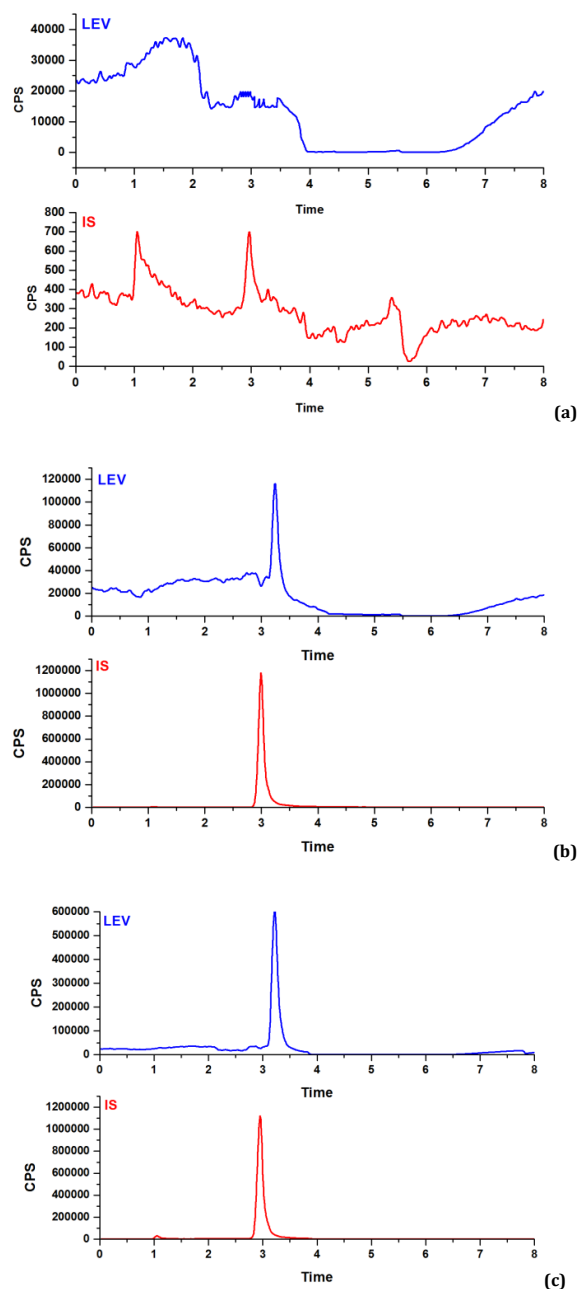
Figure 2. (a) Representative extracted ion chromatogram (XIC) of blank plasma; (b) calibration standard samples at LLOQ level and (c) calibration standard samples at ULOQ level.

The samples were stored at -80 °C between freeze/thaw cycles, and then they were thawed by allowing them to stand at room temperature for approximately 30 min. The samples were then returned to freezer for 24 h.

2.8. Application

This method was applied to monitor plasma level of LEV in single case study for patient taking toxic dose of LEV. Patient's samples (plasma) were stored at -80 °C until analysis. All patient identifiers had been removed.

3. Results

3.1. LC-MS/MS optimization

The protonated forms of the analyte and internal standard molecule [M+H]+ (m/z 170.9) and (m/z 267.3), respectively, were found to be the dominate ion in Q1 scan and were used to generate a product ion spectrum. For MRM, the most abundant fragment ion of the analyte and internal standard (m/z 140.8) and (m/z 145.0), respectively, was selected and the fragmentation conditions were optimized. Chromatographic separation was obtained with good resolution between (LEV) and IS. There is no interference between plasma ingredients and analyte as well as IS, Figure 2a-c.

Table 1. Intra- and inter-day accuracy (% of nominal concentration) and precision (%RSD) of LEV in human plasma.

Nominal concentration (ng/mL)	Intra-day accuracy (%)	Inter-day accuracy (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)	Intra-day precision (Mean±SD)	Inter-day precision (Mean±SD)
5.0	102.7	97.7	6.7	4.6	102.7±6.88	97.7±4.49
37.5	102.8	104.7	9.2	11.6	102.8±9.45	104.7±12.1
80.0	102.4	112.6	9.4	1.4	102.4±9.6	112.6±1.57

Table 2. Stabilities of LEV in human plasma quality control samples (n = 3).

Analyte	Nominal concentration (ng/mL)	% Loss/gain in stability study (Accuracy)		
		Post-preparative	Freeze-thaw	Short term
LEV	5.0	-11.2%	+3.9%	-2.5%
	37.5	-10.5%	+2.0%	+1.4%
	75.0	+4.0%	+13.4%	+1.2%

3.2. Method validation

3.2.1. Selectivity and matrix effect

Six different batches of blank human plasma were screened for any false positive MS responses. No significant peak or response was detected at the retention time for analyte and IS (S/N ratio was less than 3:1). In addition, no obvious matrix effect was found when comparing peak area of LEV in spike-after preparation samples with that in neat solution.

3.2.2. Linearity and LLOQ

Calibration curves were linear over a concentration range of 1-100 μg/mL for LEV in human plasma with an average correlation coefficient of 0.996±0.00257 (n = 3). Deviations of measured from nominal concentrations were between -1.5 to 4.5% for LLOQ (1 μg/mL) and between -11 to 10% for the other concentration levels. Precision was 3.0% at LLOQ and between 2.5 and 8.9% at the remaining concentration levels.

3.2.3. Accuracy and precision

Table 1 summarizes assay performance data for LEV in human plasma. The intra- and inter-day precision (%RSD) as well as were accuracy in the range1.4-11.6% and 97.7-112.6% for precision(%RSD) and accuracy respectively for all QC levels, thus meeting the requirement of within ±15% [21].

3.2.4. Stability test

As shown in Table 2, LEV plasma samples were stable up to 3 hours in room temperature, up to 24 hrs after sample preparation in the autosampler and after three freeze/thaw cycles as the relative deviation were within ±15% for all analytes at different concentrations.

3.3. Application

The developed method was used for determination of LEV concentration in plasma after oral ingestion. Blood have been collected at different time intervals. Plasma concentration of LEV confirms that calibration range of our method suitable for toxicological testing of LEV.

4. Discussions

The main use of guard column is to protect the analytical column from damage without notably changes in analyte retention time. In this study guard column was used to clean plasma extract to obtain more sensitivity of determination of LEV in plasma. Guard cartridge is a relatively inexpensive consumable; this will allow the replacement of it regularly to keep the performance of assay. Based on the chemical structure, electrospray ionization was used for ion generation. A Q1 full scan of LEV was performed in both positive and negative mode. The positive mode was selected due to a greater sensitivity. The utilization of Multiple Reaction Monitoring (MRM) scan mode allow determination of certain compounds in presence of interfering compounds in plasma by tandem mass [22].

Good IS should resemble the analytes during extraction and compensate for analytes on the column, especially with LC-MS/MS, due to matrix effects could produce poor analytical results. Several compounds, such as metformin, phenibut and atenolol were examined; we found that atenolol was the best. During method development different mobile phase compositions were tested for chromatographic separation of LEV and IS. In order to decrease ionization of LEV to allow more retention on column and good separation we use at pH = 2.5 (using 0.1% formic acid). Acidification of mobile phase by formic acid enhance protonation of LEV reduce the retention time and improve the peak shape.

5. Conclusion

In summary, I have developed a rapid, accurate and robust LC-MS/MS method for quantification of LEV in human plasma. The simplicity and accuracy of this make it suitable for toxicological studies as well as therapeutic drug monitoring of LEV. The uses of guard column as sample cleanup improve the performance of assay; allow quantitative determination of LEV in human plasma.

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