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Quantification of levetiracetam in human plasma by liquid chromatography–tandem mass spectrometry: Application to therapeutic drug monitoring

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ABSTRACT

A rapid, selective, reliable, precise, accurate, and reproducible tandem mass spectrometric (MS-MS) method for the quantification of levetiracetam (LEV) in human plasma using adenosine as an internal standard (IS) has been developed and validated. The drug and IS were extracted by solid phase extraction (SPE) technique and analyzed on Symmetry® C_{18} column (5 μm, 3.9 mm × 50 mm) using a mobile phase of methanol–water–formic acid (97:03:0.25, v/v/v) at a flow rate of 0.2 ml/min. Quantitation was achieved using a positive electrospray ionization (ESI+) interface employing multiple reaction monitoring (MRM) mode at MRM transitions m/z 171 > 126 and m/z 268 > 136 for LEV and IS, respectively. The method was validated over the concentration range of 1.0–40 μg/ml (r > 0.99) with a limit of quantification of 1.0 μg/ml (R.S.D.%; 4.1 and Bias%; −9.0 to + 11.0%). Intra- and inter-run precision of LEV assay at three concentrations ranged from 0.6 to 8.9% with accuracy (bias) varied from −4.0 to 8.6% indicating good precision and accuracy. Analytical recoveries of LEV and IS from spiked human plasma were in the range of 91.7–93.4% and 80.2–84.1%, respectively. Stability of LEV in human plasma samples at different conditions showed that the drug was stable under the studied conditions. Matrix effect study showed a lack of matrix effect on mass ions of LEV and IS. The described method compared well with the commercial HPLC-UV method of Chromsystem (r^2 = 0.99). The suitability of the developed method for therapeutic drug monitoring was demonstrated by measuring LEV in human plasma samples of epileptic patients treated with LEV.

1. Introduction

Levetiracetam (LEV), (*S*)-α-ethyl-2-oxo-1-pyrrolidine acetamide, is structurally unrelated to existing antiepileptic drugs (AEDs) [1]. The precise mechanism by which LEV exerts its antiepileptic effect has not been fully understood. However, it has been suggested that LEV binds with protein SV_2A in synaptic vesicles, thus reducing electrical activity in epileptic circuits [2]. LEV is indicated as adjunctive therapy in the treatment of partial seizures, with or without secondary generalization that are refractory to other established first-line AEDs [3]. Following oral administration, LEV is rapidly and almost completely absorbed from the small intestine into the systemic circulation with peak serum levels occurring at 1 to 2 h. The extent of LEV bioavailability is not affected by food. However, the rate of its absorption is delayed [4]. LEV is not significantly plasma protein bound (<10% bound) and approximately 27% of the administered dose is metabolized by enzymatic hydrolysis in the blood to inactive metabolites [4,5]. About 66% of the administered LEV dose is excreted as unchanged drug by the kidney via glomerular filtration [6].

Several chromatographic assays have been reported for the measurement of LEV in biological fluids. These involve gas chromatography (GC) with nitrogen-phosphorus detection [7], high performance liquid chromatographic (HPLC) techniques [8–12], GC–MS [13]. Most of the reported methods lack selectivity, sensitivity, and reliability. Moreover, they encounter problems particularly tedious and time-consuming sample preparation as well as high sample volume. Recently, liquid chromatography–tandem mass spectrometry (LC–MS-MS) is considered a gold standard to utilize in analysis of drugs in biological fluids. The high sample throughput, selectivity and sensitivity for analytes of interest increase the applicability of tandem mass spectrometry in clinical chemistry as well as clinical studies. In this regard, only two methods are available for analysis of LEV using tandem mass spectrometry [14,15]. The drawback of Jain's method is the utilization of clonazepam as an internal standard which would potentially create many problems involving quantification of LEV concentrations since clonazepam could concomitantly be administered with LEV to epileptic patients [14].

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However, this may lead to internal standard overestimation and consequently underestimation of LEV concentrations in the patient sample. Moreover, Jain's method has other disadvantages including large plasma sample volumes used (200 μl), high flow rates (500 μl) of the mobile phase, maintaining a column at high temperatures (45 °C) and an autosampler at low temperatures (5 °C) in addition to a tedious and time-consuming solid phase extraction (SPE) sample pre-treatment procedure. On the other hand, Guo's method did not address the matrix effect issue [15]. This is critical in establishing reliable assay method. It has been previously reported that ion suppression effects of extracted biological matrix caused by polar and un-retained matrix components were greatest after protein precipitation [16]. Thus, Guo's method lacks reliability since it did not assess the potential of matrix effect.

The objective of the present report was to develop and validate a rapid, reliable, and accurate electrospray MS-MS method for the determination of LEV in human plasma. The ion suppression/enhancement effect of the biological matrix on the MRM detection of mass ions of the analytes is to be investigated as well. The present method has been successfully utilized in therapeutic drug monitoring of LEV by analysis of plasma samples of patients treated with LEV.

2. Experimental

2.1. Chemicals and reagents

Levetiracetam was kindly supplied by UCB Pharma S.A. (Bruxel, Belgium). The internal standard (IS), adenosine was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Water was purified using a Milli-Q water device (Millipore, Bedford, MA, USA). Human plasma was kindly donated by the Central Blood Bank, Ministry of Health, Kuwait. The Oasis® HLB solid phase extraction cartridges were purchased from Waters Corporation (Milford, MA, USA). All other chemicals and reagents were of analytical grade and solvents were of HPLC grade.

2.2. Instrumentation

The chromatographic system, Waters Alliance 2690, consisted of a solvent delivery system, and an autosampler (Waters Assoc., Milford, MA, USA). Separation of the analytes was performed on Symmetry® C_{18} column (5 μm, 3.9 mm × 50 mm) and a guard column of the same material. The mobile phase used consisted of methanol–water–formic acid (97:03:0.25, v/v/v) and delivered at a flow rate of 0.2 ml/min to a positive electrospray ionization interface (ESI+) of a tandem triple quadrupole mass spectrometer (Quattro LC, Micromass, Manchester, UK). Tuning parameters of MS and MS-MS were optimized by direct infusion of solutions of LEV and the IS in the mobile phase into the ionization probe at a flow rate of 10 μl/min using a syringe pump. The ion source and desolvation temperatures were set at 120 and 350 °C, respectively. The capillary voltage was adjusted at 3.5 kV, cone voltage at 10 V, collision energy at 14 eV and collision gas pressure at $<1.0\,e^{-4}$ mbar. The MRM transitions at m/z 171 > 126 and m/z 268 > 136 were selected for quantification of LEV and IS, respectively. The data were processed by employing MassLynx NT Software (Version 4.1, Micromass, Manchester, UK).

2.3. Standard solutions, calibration standards and quality control samples

A stock solution of LEV was prepared by dissolving 10 mg of LEV powder in 10 ml methanol. A 1.0 ml aliquot of LEV stock solution (1.0 mg/ml) was diluted with 1.0 ml water to give a working standard solution of 0.5 mg/ml. The stock solution of the IS was prepared by dissolving 10 mg of the IS in 10 ml water. This stock solution (1.0 mg/ml) was further diluted in water to yield a working standard solution of 10 μg/ml and then stored at −20 °C pending analysis. The calibration standards of LEV were prepared by spiking drug-free human plasma with LEV working solution at concentrations of 1, 5, 10, 20, 30 and 40 μg/ml. Similarly, quality control (QC) samples were prepared in drug-free human plasma at concentrations of 2.5, 15 and 35 μg/ml. The spiked plasma samples were aliquoted (150 μl) into Eppendorf polypropylene tubes and kept frozen at −80 °C pending analysis.

2.4. Assay procedure

Prior to assay, frozen human plasma samples including calibrators, QC samples or patient samples were thawed at ambient temperature and then vortex-mixed for 30 s before extraction. The extraction procedure was carried out using Oasis® HLB SPE cartridges. For each sample, a SPE cartridge was conditioned with 2 ml methanol and then equilibrated with 2 ml water. A 100 μl aliquot of each plasma sample followed by 50 μl of IS (10 μg/ml) were loaded onto the activated cartridges and mixed gently for 30 s. After loading, the cartridges were then washed with 2 ml water. LEV and the IS were then eluted with 500 μl methanol under a vacuum of 5 mm Hg into a clean glass test tube. A 10 μl of the eluate was then transferred to the autosampler and injected into the LC–MS-MS system.

2.5. Assay validation

The present assay procedure using MS-MS was performed in accordance to the standard guidelines [17,18].

2.5.1. Linearity

The linearity of the proposed method was investigated by spiking LEV in drug-free human plasma at six non-zero calibration standards covering the range of 1.0–40 μg/ml and then analyzed in replicates of nine. The slope, intercept, and correlation coefficient (r) were determined by the least squares linear regression model. The various parameters of regression equation were automatically calculated by the quantifying program of MassLynx software. The lowest limit of quantification (LLOQ) was calculated on the basis of the lowest concentration of LEV that gives R.S.D.% and Bias% values ≤20%.

2.5.2. Accuracy (bias) and precision

Quality control (QC) samples at concentrations of 2.5, 15 and 35 μg/ml; covering the low, medium and high ranges of the calibration standards; were assayed in sets of replicates to assess intra-and inter-run precision and accuracy. The intra-run precision was determined from ten replicate analyses of QC samples from one calibration curve batch in 1 day. On the other hand, the inter-run precision was determined over a period of 4 weeks. The R.S.D.% serves as a measure of precision and percent deviation from the nominal concentration (Bias%) serves as a measure of accuracy.

Moreover, accuracy and precision were assessed on LEV sample concentrations above the upper limit of quantification (ULOQ), the concentration of the highest calibration standard. In such cases, the samples were appropriately diluted with drug-free human plasma to give concentrations within calibration standard. Over the range dilution experiment was carried out by preparing three concentrations of LEV in drug-free human plasma: 60, 100 and 150 μg/ml. The prepared samples were then diluted with drug-free human plasma to 1:1, 1:4, and 1:9 dilutions, respectively. Analysis of the diluted samples was conducted in a set of 6 replicates for each

diluted concentration and the accuracy (bias) and precision were then determined.

Alternatively, the accuracy and precision were investigated on low human plasma volumes containing LEV (50 μl) at concentrations of 2.5, 15 and 35 μg/ml. Each sample was diluted with drug-free human plasma to give a final volume of 100 μl. Consequently, analysis of the diluted samples was carried out employing 6 replicates for each diluted concentration and the accuracy and precision were then determined.

2.5.3. Selectivity

The selectivity of the present method was assessed by analysis of six independent sources of drug-free human plasma including heparinized, hemolyzed and lipemic plasma samples for potential interferences with endogenous compounds. The mass detector response (peak area) at the retention times of LEV and IS was compared to that of the spiked plasma at LLOQ. In addition, the potential interferences from other common antiepileptic drugs and/or their metabolites were also investigated.

2.5.4. Stability

LEV QC samples were prepared at three different concentrations (2.5, 15 and 35 μg/ml) in human plasma. Stability of LEV in human plasma was assessed through five freeze-thaw cycles from −80 °C to room temperature. Frozen plasma samples (QC) were allowed to stand at room temperature for 2 h to allow complete thawing before being processed for analysis. Alternatively, stability of LEV samples in the autosampler was investigated by injecting the same processed LEV samples over 24 h at ambient temperature.

Moreover, the effect of frozen storage on LEV stability in human plasma was evaluated through storing of LEV plasma samples at −80 °C over a period of 1 month. LEV plasma samples were analyzed immediately after spiking and at selected time intervals after storage over the storage period. Stability was defined as <10% loss of initial drug concentration.

2.5.5. Recovery and matrix effect

The matrix effect (ME) study was assessed employing two approaches. The first approach was performed during the assay method development procedure using a postcolumn infusion protocol [16]. In this study however, a 10 μg/ml solution of either LEV or IS was continuously infused into the column effluent via postcolumn "tee" connection using a syringe pump and then an aliquot of 10 μl of drug-free human plasma was analyzed by HPLC. The MRM intensities of LEV and IS at m/z 171 > 126 and m/z 268 > 136, respectively, were used for monitoring ion suppression/enhancement.

Alternatively, the ME was further investigated, during the validation procedure of the present assay method, employing Matuszewski method [19]. The protocol however, was performed by determination of peak areas of LEV in three different sets of samples, one consisting of pure standards in methanol (set 1), one prepared in drug-free human plasma (blank matrix) extracts from six different sources and spiked with LEV and IS after extraction (set 2), and one prepared in blank matrix from the same sources but spiked before extraction (set 3). From these data the ME was then calculated as a percentage of the response of set 2 samples in relation to those of set 1 samples, the recovery as a percentage of the response of set 3 samples in relation to that of set 2 samples, and finally the process efficiency as a percentage of the response of set 3 samples in relation to that of set 1 samples [19].

The ME, recovery (RE) of the extraction procedure, and overall "process efficiency" (PE) were determined by comparing the absolute peak areas for analytes obtained in sets 1–3 as follows:

$$\text{ME } (\%) = \frac{B}{A} \times 100$$

$$\text{RE } (\%) = \frac{C}{B} \times 100$$

$$\text{PE } (\%) = \frac{(\text{ME} \times \text{RE})}{100},$$

where, A is the peak areas obtained in pure solution standards in set 1, B is the corresponding peak areas for standards spiked after extraction into plasma extracts (set 2), and C is the peak areas for standards spiked before extraction (set 3) [19].

Absolute (extraction) recovery of LEV from human plasma was evaluated using QC samples (2.5, 15, and 35 μg/ml). Recovery was determined by comparing the peak areas obtained from plasma samples with the analytes spiked before extraction to those spiked after extraction.

2.5.6. Method comparison

The proposed LC–MS-MS assay method was compared with the commercial HPLC-UV method of Chromsystem (München, Germany) by analyzing LEV in patient samples employing the two methods. For the comparison, aliquots from 37 patient plasma samples were processed and analyzed by both methods.

2.5.7. Clinical application

The clinical applicability of the present method was evaluated by analysis of LEV in plasma samples refereed to our TDM-CT laboratory for routine monitoring of LEV.

The following equation was used to calculate LEV oral clearance (CL/F):

$$\frac{\text{CL}}{F}(\text{ml/min/kg}) = \frac{\text{Dose} \times 1000}{C_{ss} \times 1440}$$

where, dose is the LEV dose (mg/kg/day), C_{ss} is the steady-state LEV trough plasma concentration (μg/ml), and F is the oral bioavailability.

3. Results and discussion

3.1. Analysis conditions

Tandem mass spectrometry (MS-MS) is being utilized in our TDM-CT laboratory for analysis of drugs and/or metabolites in biological matrices. The technique provides specificity because of its ability to monitor selected mass ions, sensitivity because of the enhanced signal-to-noise ratio, and speed because it can help avoid the need for tedious sample cleanup and lengthy analysis times. This is of high significance in achieving good selectivity and sensitivity to permit very fast analytical method separation and achieve high sample throughput. In order to undertake successful quantification of LEV, the appropriate tuning parameters for ESI+ were optimized for detection of the protonated parent and daughter ions of LEV and IS. Fig. 1 shows the parent/daughter ions of LEV at m/z 171 > 126, whereas Fig. 2 exhibits the parent/daughter ions of IS at m/z 268 > 136. Separation of the analytes from human plasma was achieved using solid phase extraction techniques. A mobile phase consisting of methanol–water–formic acid (97:03:0.25, v/v/v) was found optimal since it enhances the formation of the parent and fragment ions of LEV and IS. However, after trying many different kinds of reverse phase C_{18} columns, it was found that Symmetry®

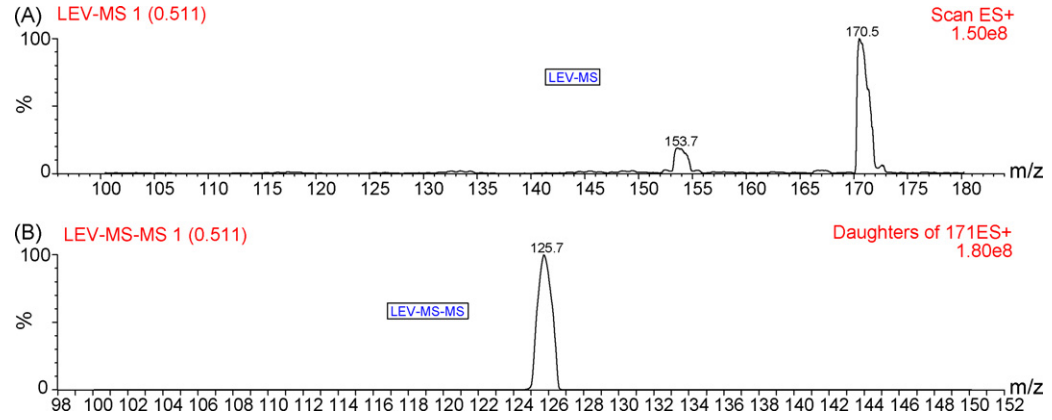

Fig. 1. MS [A] and MS-MS [B] scans of LEV.

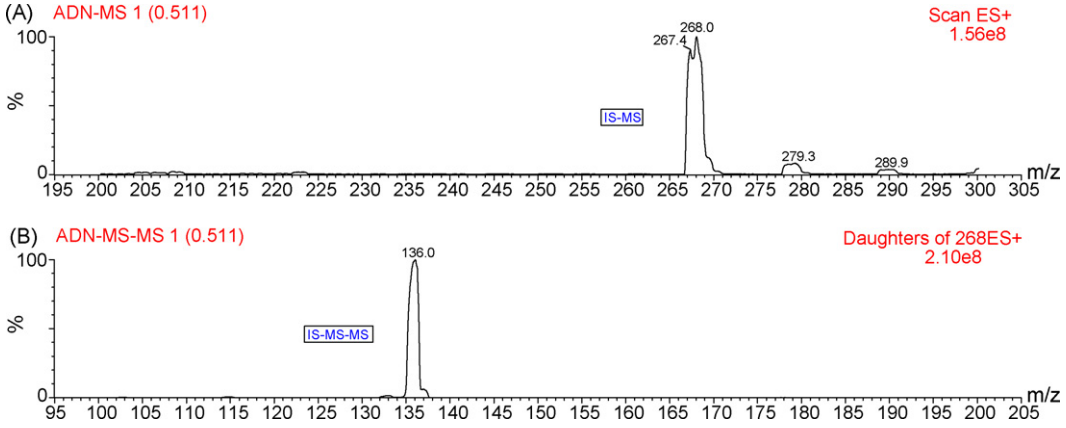

Fig. 2. MS [A] and MS-MS [B] scans of internal standard (IS).

C_{18} column (5 μm, 3.9 mm × 50 mm) produced the best chromatographic results in terms of peak shape and retention of analytes. Moreover, under the selected experimental conditions, the analysis run cycle-time was approximately 5 min injection–injection. This is important when a large number of patient samples have to be analyzed. On the other hand, sample pre-treatment in the proposed method was undertaken using SPE technique without evaporation of the sample to dryness. Although matrix clean up is more extensive with SPE, the dilution without pre-concentration step and direct injection of the sample, however, had much less ion suppression in LC–MS-MS as observed in the present method.

3.2. Method validation

3.2.1. Linearity

Linearity of LEV assay was established over a concentration range of 1.0–40 μg/ml in spiked human plasma. The selected standard calibration range covers the expected tentative therapeutic plasma levels of LEV in samples of epileptic patients. Linear correlations ($r > 0.99$) were obtained using least squares linear regression model using peak area ratios with the LLOQ of 1.0 μg/ml. The linear regression equation obtained was: $y = -0.042 + 0.091x$; $n = 9$, where y is peak area ratio of LEV to the IS and x is the LEV concentration, expressed as μg/ml. Accuracy and precision at LLOQ were within the normal limits (R.S.D.%; 4.1% and Bias%; −9.0 to +11.0%).

3.2.2. Selectivity

Selectivity is the ability of the analytical method to measure and differentiate the analyte in the presence of endogenous and/or exogenous components. Fig. 3 demonstrates typical MRM chromatograms of a drug-free human plasma whereas Fig. 4 shows the MRM chromatograms of a drug-free human plasma spiked with LEV and IS. On the other hand, the typical MRM chromatograms of a plasma sample of a patient on LEV therapy are presented in Fig. 5.

The present method however, established good selectivity as demonstrated by lack of interfering peaks at the retention times of either LEV or IS. This was proved by investigating six different lots of drug-free human plasma samples. None of the tested lots of plasma showed any interference at the retention times of LEV or

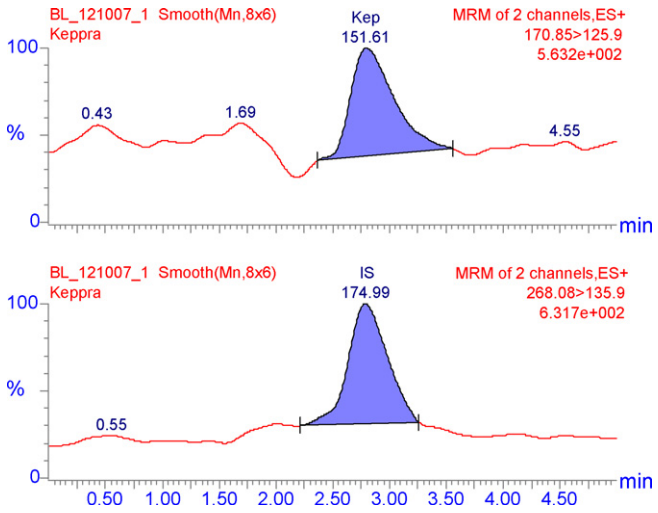

Fig. 3. Typical MRM chromatograms of a drug-free human plasma.

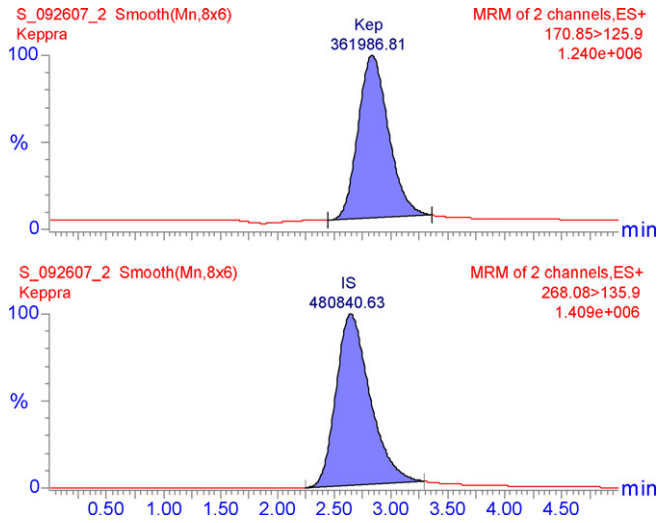

Fig. 4. Typical MRM chromatograms of a drug-free human plasma supplemented with LEV (upper) and IS (lower) [LEV concentration: 5.0 μg/ml].

IS. Moreover, the mass detector response at the retention times of LEV and the IS was compared to the LLOQ. The area observed at the retention time of LEV was less than 7% at the LLOQ area, whereas the area observed at the retention time of the IS was less than 1.0% of the area of the IS level used in the present method.

On the other hand, the specificity of the developed method was investigated by examining the potential interferences of co-administered antiepileptic drugs under the established analytical conditions. In this regard, carbamazepine, carbamazepine-10,11-epoxide, oxcarbazepine, 10-hydroxy-carbazepine, topiramate, lamotrigine, felbamate, vigabatrin, gabapentin and zonisamide exhibited no interferences with LEV determination.

3.2.3. Accuracy and precision

The data on accuracy (bias) and precision of the present assay are shown in Table 1. The intra-run accuracy (bias) ranged between −1.5 and 8.6% with a precision of 0.6–2.7% while the inter-run accuracy varied between −4.0 and −0.8% with a precision of 4.8–8.9%. The results of the present method demonstrated adequate precision and accuracy.

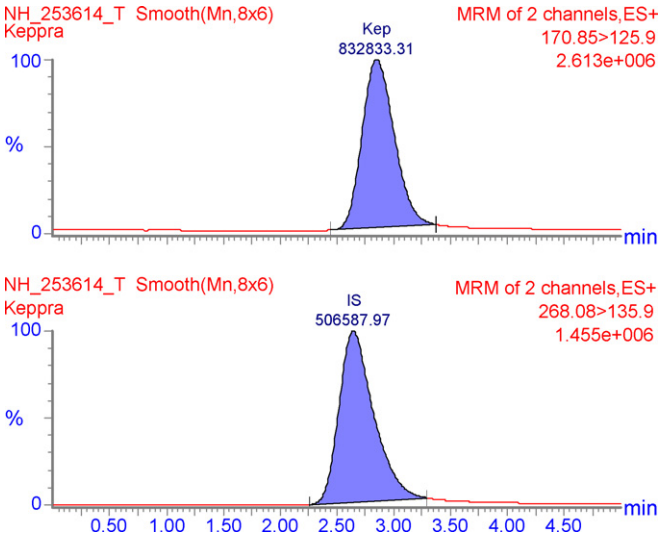

Fig. 5. Typical MRM chromatograms of a patient plasma sample taken at steady-state prior to an LEV dose of 1500 mg/day [LEV (upper) and IS (lower)], LEV concentration = 11.1 mg/l.

Table 1
Intra- and inter-run precision and accuracy for determination of LEV in human plasma by LC–MS-MS

Nominal concentration (μg/ml)	Found (mean ± S.D.) (μg/ml)	R.S.D.%	Bias%*
Intra-run[a]			
2.5	2.56 ± 0.07	2.73	2.40
15	16.29 ± 0.38	2.32	8.60
35	34.49 ± 0.19	0.55	−1.46
Inter-run[b]			
2.5	2.48 ± 0.22	8.88	−0.80
15	14.40 ± 0.99	6.85	−4.00
35	33.99 ± 1.63	4.78	−2.89

[a] $n = 10$.
[b] Precision and accuracy (bias) were determined from ten different runs over a 4-week period for each concentration.
* Bias% = 100 × (Found concentration − Nominal concentration/Nominal concentration).

3.2.4. Dilution precision

Over the range dilution study of LEV which was performed at 1:1, 1:4 and 1:9 dilution showed that dilution of LEV samples above ULOQ can be undertaken with good precision and accuracy. The precision of the study ranged from 4.2 to 8.0% and accuracy (bias) ranged between −4.0 and 1.5%.

Alternatively, the results of low plasma volume underwent 1:1 dilution demonstrated adequate precision and accuracy. The precision of the study varied between 4.5 and 7.0% and accuracy ranged between −1.7 and 5.7%.

3.2.5. Stability

Table 2 shows the results of LEV stability study involving the processed samples in the autosampler, freeze-thaw and long-term storage (−80 °C). The processed LEV samples kept in the autosampler (at ambient temperature) demonstrated that the samples were stable for up to 24 h. Alternatively, the results of LEV stability study after five freeze-thaw cycles showed that the drug was stable for at least five freeze-thaw runs. Moreover, the results of frozen storage on LEV stability indicated that the drug was stable for at least 4 weeks when kept frozen at −80 °C with no appreciable degradation products. The data of the stability study however, demonstrated that LEV samples were stable under the tested conditions and suggest that the collected patient samples can safely be stored for TDM/PK purposes.

3.2.6. Recovery and matrix effect

MS encounters some problems such as ion suppression which results from the presence of less volatile compounds that can change the efficiency of the assay because it affects the amount of charged ion in the gaseous phase that ultimately reaches the mass

Table 2
Summary of LEV stability study in human plasma

Nominal concentration (μg/ml)	2.5	15	35
Autosampler at 25 °C (24 h)			
Mean concentration found ($n = 5$)	2.8	14.5	33.9
R.S.D.%	3.0	0.8	0.7
Bias%	12.8	−3.3	−3.1
Freeze-thaw			
Mean concentration found ($n = 5$)	2.6	14.4	34.3
R.S.D.%	6.2	3.3	1.6
Bias%	5.2	−3.7	−2.1
Long-term at −80 °C (30 days)			
Mean concentration found ($n = 5$)	2.5	14.7	35.4
R.S.D.%	9.8	4.9	5.8
Bias%	0.8	−1.9	1.1

Table 3
Precision[a] (R.S.D.%) and accuracy (Bias%) of determination of peak areas of LEV (1), IS (2), and the peak area ratios (1/2) in sets 1[b], 2[c], and 3[d] using ESI

Nominal concentration (mg/l)	Precision (R.S.D.%)									Bias%[e]
	Peak area-1			Peak area-2			Peak area ratio (1/2)			
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	
2.5	1.56	5.8	2.41	0.75	5.56	8.38	1.32	5.36	4.51	1.33
15	0.9	7.27	8.55	2.14	4.00	4.77	1.63	7.18	1.59	3.11
35	0.32	3.29	7.49	1.04	2.97	9.22	0.68	2.35	2.35	−1.9

[a] $n = 6$.
[b] LEV and IS standards in methanol.
[c] LEV and IS spiked after extraction into extracts from six different plasma lots.
[d] LEV and IS spiked before extraction into extracts from six different plasma lots.
[e] Bias = (Mean observed concentration − Nominal concentration/Nominal concentration) × 100.

detector [20]. Suppression or enhancement of analyte ionization by co-eluting compounds is a well-known phenomenon in LC–MS-MS mainly depending on the sample matrix, sample preparation procedure, quality of chromatographic separation, mobile phase additives, and ionization type [18,19,21]. It is obvious that ion suppression may affect validation parameters such as LLOQ, linearity, precision and/or bias.

For methods using LC–MS, experiments for assessment of potential matrix effects, i.e., ion suppression or ion enhancement, should always be part of the validation process, particularly if they employ ESI, and lack of ion suppression effects due to extracting plasma constituents on the measured mass ions using electrospray tandem mass spectrometry is mandatory [16,18,19].

The ME was initially performed by postcolumn infusion experiment during the method development procedure and consequently the separation system was optimized [18]. The conditions of the present assay method optimize the separation of LEV and IS in a region where ion suppression is not observed. Moreover, during the validation procedure the second approach was performed [19].

The relative ME of the analytes was evaluated by comparing the peak areas of LEV and IS spiked into extracts of 6 independent drug-free human plasma lots. The results of precision of determination of LEV and IS responses employing the described method are shown in Table 3. The precision of determination of LEV and IS peak areas at the three concentrations of QC samples varied from 3.3 to 7.3% and 3.0 to 5.6%, respectively. This precision is slightly higher than that of determination of standards injected directly in methanol (0.3–1.6% and 0.8–2.1%, respectively. On the other hand, the precision of determination of LEV and IS spiked before extraction varied from 2.4 to 8.6% and 4.8 to 9.2%, respectively. These data however, confirm a lack of relative ME for LEV and IS employing the proposed method.

The results of ME on LEV and IS are presented in Table 4. The ME as mean value was of 104.1 and 100.5% for LEV and IS, respectively. The ME assessment shows no absolute matrix effect was observed from the matrix of human plasma employed in the present study.

Recoveries of LEV from spiked human plasma samples were in the range of 91.7–93.4% whereas that of the IS were 80.2–84.1%, indicating the suitability of SPE procedure for separation of LEV and IS from human plasma and lack of ion suppression effect.

3.3. Method comparison

The described assay method compared well with the commercial HPLC-UV method provided by Chromsystem, $r^2 = 0.99$. The linear regression equation was: LC–MS-MS = −0.05 + 1.02HPLC, $n = 37$. Moreover, the proposed method is monthly assessed by Heathcontrol for external quality control assessment (Cardiff Bioanalytical Services, Cardiff, UK). The monthly reports however, demonstrated that the present method correlates well with the mean consensus values for LEV determination in plasma.

3.4. Clinical application

Our TDM-CT lab is routinely analyzing AEDs in plasma samples of epileptic patients. Currently, the proposed assay method is routinely employed in the analysis of plasma samples of epileptic patients treated with LEV.

The relationship between LEV daily dosage and steady-state trough concentration in 44 patients on LEV therapy is shown in Fig. 6. The trough plasma concentrations of LEV were in the range of 2.9–31.7 μg/ml (mean ± S.D.; 14.7 ± 7.1 μg/ml). LEV trough plasma concentrations were linearly related to daily drug doses ($r = 0.45$). However, a large inter-individual variability in LEV concentrations was observed within the same drug dosage and this concurs with earlier reports [22]. On the other hand, the relationship between age and LEV trough concentration demonstrated a lack of correlation between LEV trough concentrations and age ($r = 0.07$).

Table 4
Marix effect (ME), recovery (RE), and process efficiency (PE) data for LEV (1), IS (2), in six different lots of human plasma using ESI

Nominal concentration (mg/l)	Mean peak area[a]						ME[b] (%)		RE[c] (%)		PE[d] (%)	
	LEV			IS								
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	1	2	1	2	1	2
2.5	5.44	5.62	5.24	86.42	80.38	67.47	103.24	93.02	93.42	84.06	96.45	78.20
15	65.53	69.7	65.54	77.15	82.5	66.11	106.35	107.00	91.67	80.17	97.49	85.78
35	156.64	160.72	147.62	77.59	78.7	64.08	102.6	101.44	91.83	81.51	94.22	82.68

[a] In arbitrary units, $\times 10^4$, $n = 6$.
[b] Matrix effect expressed as the ration of the mean peak area of an analyte spiked postextraction (set 2) to the mean peak area of the same analyte standards (set 1) multiplied by 100. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression.
[c] Recovery calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of an analyte spiked postextraction (set 2) multiplied by 100.
[d] Process efficiency expressed as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of the same analyte standards (set 1) multiplied by 100.

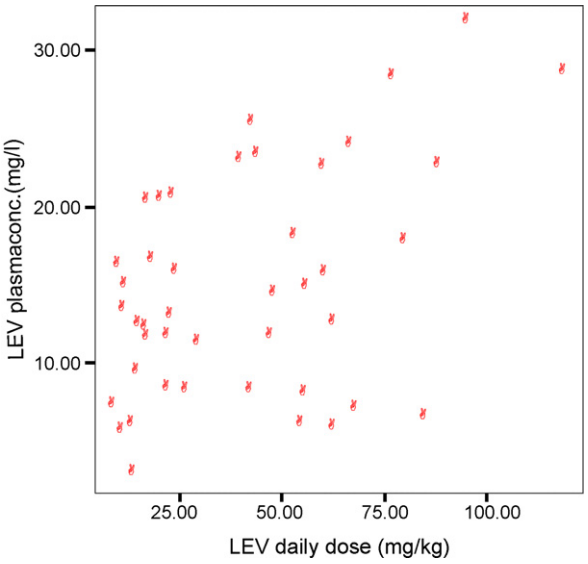

Fig. 6. Relationship between LEV daily dose (mg/kg) and LEV steady-state trough levels ($r = 0.45$; $n = 44$).

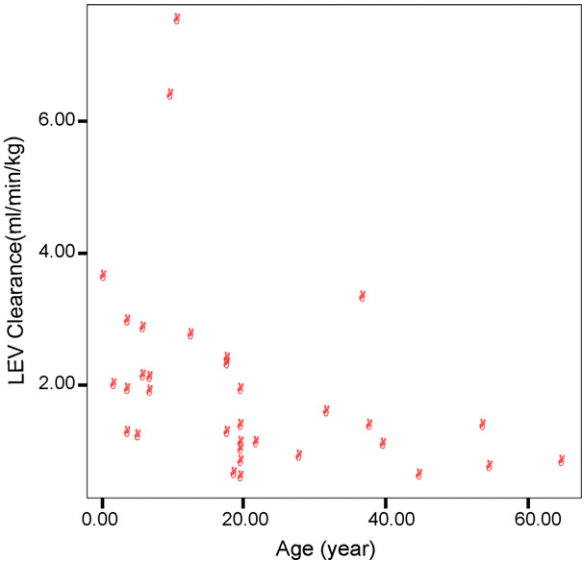

Fig. 7. Relationship between LEV oral clearance and patient's age.

However, as shown in Fig. 7, children demonstrated high mean LEV oral clearance values in contrast to adults (2.92 ± 1.91 ml/min/kg vs 1.27 ± 0.70 ml/min/kg) indicating that the children may require high LEV dosage (mg/kg) than adults to achieve optimal clinical responses.

4. Conclusion

An accurate, precise, reliable, and specific LC–MS-MS method for determination of LEV in human plasma is described. The developed method verified that, with SPE procedure, chromatographic separation, and MS conditions selected for the assay, the matrix effect for LEV and the IS was not observed as well as absence of "cross-talk" effect. The present method however, is suitable for routine analysis of LEV in plasma samples of patients to monitor their therapeutic or toxic levels as well as for pharmacokinetic studies. The described method is routinely employed in our TDM-CT lab for measurement of LEV in plasma samples of epileptic patients taking the drug.

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References

[1] S. Shorvon, Lancet 358 (2001) 1885–1892.
[2] B. Lynch, N. Lambeng, K. Nocka, P. Kensel-Hammes, S. Bajjalieh, A. Matagne, B. Fuks, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 9861–9866.
[3] J. Cereghino, V. Biton, B. Abou-Khalil, F. Dreifuss, L. Gauer, I. Leppik, Neurology 55 (2000) 236–242.
[4] P. Patsalos, Pharmacol. Ther. 85 (2000) 77–85.
[5] M. Strolin Benedetti, R. Whomsley, J. Nicolas, Eur. J. Clin. Pharmacol. 59 (2003) 621–630.
[6] M. Walker, P. Patsalos, Pharmacol. Ther. 67 (1995) 351–384.
[7] T. Vermeij, P. Edelbroek, J. Chromatogr. Biomed. Appl. 662 (1994) 134–139.
[8] N. Ratnaraj, H. Doheny, P. Patsalos, Ther. Drug Monit. 18 (1996) 154–157.
[9] V. Pucci, F. Bugamelli, R. Mandrioli, A. Ferranti, E. Kenndler, M. Raggi, Biomed. Chromatogr. 18 (2004) 37–44.
[10] B. Rao, R. Ravi, B. Shyam Sundar Reddy, S. Sivakumar, I. Gopi Chand, K. Praveen Kumar, P. Acharyulu, G. Om Reddy, M. Srinivasu, J. Pharm. Biomed. Anal. 35 (2004) 1017–1026.
[11] J. Martens-Lobenhoffer, S. Bode-Boger, J. Chromatogr. B 819 (2005) 197–200.
[12] J. Juenke, P. Brown, F. Urry, G. McMillin, J. Anal. Toxicol. 30 (2006) 27–30.
[13] N. Isoherranen, M. Roeder, S. Soback, B. Yagen, V. Schurig, M. Bialer, J. Chromatogr. B 745 (2000) 325–332.
[14] D. Jain, G. Subbaiah, M. Sanyal, U. Pal, P. Shrivastav, Rapid Commun. Mass Spectrom. 20 (2006) 2539–2547.
[15] T. Guo, L. Oswald, D. Mendu, S. Soldin, Clin. Chim. Acta 375 (2007) 115–118.
[16] C. Müller, P. Schäfer, M. Störtzl, S. Vogt, W. Weinmann, J. Chromatogr. B 773 (2002) 47–52.
[17] V. Shah, K. Midha, S. Dighe, I. McGilveray, J. Skelly, A. Yacobi, T. Layloff, C. Viswanathan, C. Cook, R. McDowall, K. Pittman, S. Spector, Pharm. Res. 9 (1992) 588–592.
[18] V. Shah, K. Midha, J. Findlay, H. Hill, J. Hulse, I. McGilveray, G. McKay, K. Miller, R. Patnaik, M. Powell, A. Tonelli, C. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551–1557.
[19] B. Matuszewski, M. Constanzer, C. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[20] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942–950.
[21] T. Annesley, Clin. Chem. 49 (2003) 1041–1044.
[22] T. May, B. Rambeck, U. Jürgens, Ther. Drug Monit. 25 (2003) 690–699.