A Simple Bioanalytical Method for the Quantification of Levetiracetam in Human Plasma and Saliva

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Abstract

A novel, sensitive and selective ultra-high-performance liquid chromatography - coupled to electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) method was developed and validated for the quantification of levetiracetam (LEV), a broad-spectrum antiepileptic drug, in human plasma and saliva. A simple protein precipitation method with acetonitrile as precipitating solvent was used to extract LEV from human plasma and saliva. LEV and an internal standard (fluconazole, IS) were separated on a Kinetex analytical column (5 μm C18 100A, 100 × 2.1 mm) using an isocratic solvent system consisting of methanol, water and 100% formic acid at a ratio of 97:3:0.25 (v/v/v) and a flow rate of 0.2 ml/min over 2 min run time. Detection was performed using ion trap mass ions of [M+H]+ at m/z 171.0 for LEV and m/z 307.0 for IS in selective ion mode. The method was validated in the calibration range of 1.0-50.0 μg/mL for plasma and 0.5-30.0 μg/mL for saliva. Thus the present HPLC-MS/MS method for determination of LEV in human plasma and saliva, is highly sensitive, rapid with a short run-time of 2 min, can be suitable for high sample throughput. The developed method required minimal sample preparation and less plasma sample volume compared to earlier published LC-MS/MS methods. The validation parameters were found to be well within the acceptance limit.

Keywords: Levetiracetam; LC-MS/MS; Plasma; Saliva; Validation

Introduction

Levetiracetam, (S)-α-ethyl-2-oxo-1-pyrrolidine acetamide, is the (S)-enantiomer of the ethyl analogue of piracetam and shares its chemical structure with numerous nootropic drugs [1]. Levetiracetam (LEV) was developed as a new substance that acted on the central nervous system. LEV is an antiepileptic drug (AED) with a unique preclinical and pharmacological profile [2,3].

Levetiracetam has favorable pharmacokinetic parameters, including oral bioavailability of 100%, linear pharmacokinetics, minimal protein binding (10%), lack of hepatic metabolism, is not metabolized by CYP-dependent pathways, rapid achievement of steady-state concentrations, low potential for drug interactions, and a half-life of 6-8 hours [1,4]. It is primarily distributed in body water and has been shown to readily enter cerebrospinal fluid and the brain [1].

The saliva may be used as alternative matrix for drug monitoring concentration [2]. Saliva can be collected by non-invasive techniques and many samples can be obtained without exposing patients to discomfort, skin irritation and infection risk. This is of special importance for children and elderly patients. Also, when single or multiple serial samples are required from out-patients, the organization of collecting saliva samples is relatively simple, since they can be collected at home [5].

Several methods have been developed for quantification of LEV concentration in human plasma, e.g., gas chromatography (GC) with nitrogen-phosphorus detection or mass spectrometry [6], high-performance liquid chromatography (HPLC) with UV detection [7], and more recently liquid chromatography with tandem mass spectrometry (LC-MS/MS) [8] and ultra-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) [9]. A variety of analytical procedures have been developed for the determination of levetiracetam in saliva using gas chromatography (GC) with mass spectrometry [10] and high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) [11,12].

In this study a sensitive, simple and high-throughput LC-MS/MS method (optimized and validated according to EMEA/CHMP/EWP/192217/2009 guideline) was developed for the quantification of LEV concentration in small volumes of plasma and saliva. The method was used to monitor level of levetiracetam in patients of Departments of Neurology, Institute of Psychiatry and Neurology for purpose of clinical needs in epilepsy treatment. For extraction of levetiracetam from matrices a simple and direct protein precipitation with acetonitrile (ACN) with internal standard (IS) was used. Fluconazole as internal standard (IS) has been selected based on an analysis patient cards. Fluconazole was not used in these patients as a drug.

Materials and Methods

Chemicals and reagents

Levetiracetam (purity ≥ 98%) and fluconazole (purity ≥ 98%, the internal standard, IS) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol (LC-MS-grade), acetonitrile (LC-MS-grade), formic acid (LC-MS-grade) and water (LC-MS-grade) were also purchased from Sigma-Aldrich, (Steinheim, Germany). Pooled human plasma was obtained from Regional Blood Center (Warsaw, Poland). Blank human saliva samples were obtained from healthy individuals.

Instruments and chromatographic conditions

Keywords: Levetiracetam; LC-MS/MS; Plasma; Saliva; Validation

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The LC-MS/MS system used for method development and validation was performed on the liquid chromatography system (Shimadzu, Duisburg, Germany) consisting of LCMS-8030 Triple Quadrupole Liquid Chromatography Mass Spectrometer (LC-MS/MS), LC-20AD binary gradient pump, SIL-20ACXR auto-sampler, DGU-20A3 degasser and a column oven CTO-20AC (Shimadzu Corporation, Kyoto, Japan). Data acquisition and processing were conducted using the LabSolution LC-MS 5.42 SP3 software package for quantification (Shimadzu).

Chromatographic conditions were identical for plasma and saliva samples. Chromatographic separation was achieved on a Kinetex analytical column, 5 µm C18 100A, 100 × 2.1 mm (Phenomenex, Torrance, CA, USA) with a corresponding guard column Security Guard Ultra UHPLC 2.1 ID (Phenomenex, Torrance, CA, USA). The temperature of column department was set to 40°C. The mobile phase was an isocratic solvent consisting of methanol, water and 100% formic acid at a ratio of 97:3:0.25 (v/v/v) and a flow rate of 0.2 mL/min. The needle rinse solvent was methanol. The injection volume was 1 µL.

The mass spectrometry was optimized to get the highest intensity for levetiracetam. Nitrogen was used as the nebulizing gas. Argon was used as the collision gas. Instrument settings were as follows: capillary voltage 4 kV, nebulizer gas flow N2 2.0 l/min, drying gas flow N2 10.0 l/min, DL temperature 200°C. Heat Block temperature 300°C and collision energy offset of -20.0 V for levetiracetam and at -20.0 for IS.

Sample analysis was performed in the multiple reaction monitoring mode (MRM) monitoring the transition of the m/z 171.2 precursor ion to the m/z 126.0 product ion for levetiracetam (171.0→126.0) and m/z 307.0 precursor ion to the m/z 220.0 product ion for IS (307.0→220.0). Quadrupoles Q1 and Q3 were set to unit resolution. The dwell time was 3 msec, pause time was 1 msec. The mass spectrometer was operated in a positive ion mode. The mass spectrometric signal was recorded after injection, and the total analytical run time was 2.0 min. Analyst software was used to control the system, data acquisition, integrate peak area and calculate the concentration of unknowns against a standard curve derived from calibrators analyzed within the same analytical run.

Preparation of stock and standard solutions

Standard stock solution and IS stock solutions of flucloxacilone were prepared from solid powders and dissolved into methanol. Standard stock solutions (levetiracetam 1.0 mg/mL) and IS stock solutions (flucloxacilone 1.0 mg/mL) were stored at ≤ 8.0°C. Standard curve and quality control (QC) samples were prepared by diluting stock solutions with blank human plasma or saliva. A calibration curve was constructed by plotting the peak area ratio of levetiracetam to IS against the nominal analytic concentration ratio using the following calibration: 1.0, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 µg/mL of levetiracetam in plasma and 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0 and 30.0 µg/mL for saliva. Concentrations of 2.5, 15.0 and 45.0 µg/mL were chosen as the low QC level (LQC), mid QC level (MQC) and high QC level (HQC) values for plasma and 0.75, 7.5 and 25.0 µg/mL were set as the QC concentrations for saliva, respectively. IS stock solution was diluted to achieve a final concentration of 26.67 µg/mL for plasma and 13.33 µg/mL for saliva. Calibration and QC samples were freshly analyzed at the beginning of each batch of specimens and then used to determine concentrations of QC and unknown samples.

Sample preparation

Before analysis, the plasma and saliva samples were thawed to room temperature. Sample preparation was performed in a 1.5 mL polypropylene microcentrifuge tube. 20 µL matrix samples (plasma or saliva) were added to 20 µL of water and the mixture was shaken on a vortex mixer for 30 s at 1500 rpm. Plasma or saliva aliquots were deproteinized by addition 200 µL of ACN spiked with IS, vortexed for 30 s and then centrifuged at 15,000 g for 15 min. The 50 µL clear organic supernatant was transferred to the 1.5 mL polypropylene microcentrifuge tube and was reconstituted with 250 µL mobile phase. The mixture was shaken on a vortex mixer for 30 s at 1500 rpm. The solution was transferred to the autosampler vessel and 1.0 µL was injected into the LC-MS/MS system.

Data analysis

A LabSolutions LCMS 5.42 SP3 software was employed to obtain and analyze the chromatogram. Microsoft Office Excel 2007 was used to calculate intra- and inter-assay means, standard deviation (SD) and relative standard deviation (RSD), as well as coefficient of variation (CV), etc.

Method validation

Validation was based on the European Medicine Agency (EMEA) guideline - EMEA/CHMP/EWP/192217/2009. The validation was performed in order to evaluate the methods in terms of specificity, carry over, sensitivity, calibration curve (linearity of response), accuracy, precision, dilution integrity, matrix effect, recovery and stability.

Selectivity: Selectivity of the method was assessed by comparing chromatograms of blank plasma or saliva from each of six different sources was extracted without addition of any standards. Proteins were precipitated using acetonitrile without IS. The data of the chromatograms were processed and the integrated instrument of interfering compounds extracted from blank plasma or saliva response should not exceed 20% of the average integrated response of the LLOQ of LEV and 5% of the integrated response of IS.

Limit of detection (LOD) was calculated from the standard deviation (SD) of response and slope of the curve (S) using the equations: LOD=3.3 × (SD/S), according to ICH Q2 (R1) (Validation of analytical procedures: text and methodology).

Linearity: A total of six calibration lines, consisting of seven different concentrations, were prepared in blank human plasma or saliva and measured during six runs. Calibration curves were obtained by fitting the peak area ratios to a weighted (1/y) least squares regression model. Concentrations were evaluated on the basis of the corresponding calibration curve, and deviations from the theoretical concentrations were required to be within ± 20% for the LLOQ and within ± 15% for other calibration levels. Correlation coefficients (r) were required to be 0.99 or higher.

Inter-assay accuracy and precision: The intra-day and inter-day accuracy and precision were assessed by the replicate analysis (n=6) of QC samples (plasma or saliva) at three different concentrations on the same day and three consecutive days, respectively. Intra- and inter-day assay precisions were determined as % CV (coefficient of variance), and the %CV was required to not exceed 20% for the LLOQ and 15% for other concentrations. The intra-and inter-day assay accuracies were determined as the percent difference between the mean of observed concentrations and the theoretical concentration, and were required to be within ± 20% for the LLOQ and within ± 15% for other concentrations.

Recovery and matrix effect: For levetiracetam and IS, the matrix factor (MF) was measured in 6 different lots of plasma or saliva, by calculating the ratio of the peak area in the presence of matrix (measured...
by analyzing blank matrix spiked after extraction with levetiracetam),
to the peak area in absence of matrix (pure solution for levetiracetam).
The CV of the IS-normalized MF calculated from the 6 lots for each of plasma and saliva should not be greater than 15%. This determination should be done at a low and at a high QC level of concentration.

**Stability:** The stability of levetiracetam in matrix samples was assessed by analyzing replicates QC samples during the sample and storage procedure. For all stability studies, freshly prepared and stability testing QC samples were evaluated by using a freshly prepared standard curve for the measurement. The short-term stability was assessed after exposure of the matrix samples to room temperature for 24 h. The Freeze/thaw stability was determined after three freeze/thaw cycles. The concentrations obtained from all stability studies were compared to freshly prepared QC samples that were at room temperature for 4 h, and the percentage concentration deviation was calculated. In addition, long-term stability was assessed for QC samples stored at -14°C and at -60°C for 14 days. The analytes were considered stable in human plasma when the concentration difference was less than 15% between the freshly prepared samples and the stability testing samples.

**Results and Discussion**

**Mass spectrometry**

The mass spectrometer was operated in the positive ion MRM mode for both levetiracetam and fluconazole in the LC-MS/MS analysis. ESI spectra revealed higher signals at m/z 171.20 for levetiracetam. The full scan spectra was dominated by protonated molecules [M+H]^+. The product ion mass spectra of two protonated molecular ions are shown in Figure 1, in which the most abundant ions were observed at m/z 126.00 (levetiracetam). By monitoring the product ions, a highly sensitive assay for levetiracetam and fluconazole was developed. Additional tuning of ESI source and collision-induced dissociation parameters onto the transition m/z 171.20→126.00 (levetiracetam) further improved the sensitivity. Hence structurally related compound having similar chromatographic properties, mass spectrometric behavior and extraction characteristics was selected as IS. Therefore, fluconazole was chosen as IS for levetiracetam. The positive ESI mode, fluconazole predominantly formed the protonated molecule [M+ H]^+ in full-scan spectra. To determine fluconazole using the MRM mode, full-scan and product ion spectra were investigated. Representative mass spectra of [M + H]^+ ions of fluconazole is shown in Figure 1. The major fragment ions at m/z 307.00→220.00 (fluconazole) were chosen in the MRM mode.

**Liquid chromatography**

The chromatographic conditions were investigated to optimize sensitivity, speed and peak shape. Different trails were carried out for effective separation of drug and internal standard without the interference of plasma by changing the composition of mobile phase, using different columns, altering the column temperature. Analyte and IS were free of interference from endogenous substances. The Kinetex C18 column (100 × 2.1 mm, 5 µm) provided very good selectivity, sensitivity and peak shape for levetiracetam and IS as compared with other columns. The mobile phase consisting of methanol, water and 100% formic acid (97:3:0.25, v/v/v) with flow rate of 0.2 mL/min was found to be suitable during LC optimization. The retention time of levetiracetam and fluconazole was found to be 1.079 min and 1.072 min,
Figure 2: LC-MS/MS chromatograms of (A) a blank human plasma samples, (B) a plasma sample at the LLOQ level, (C) extracted blank plasma sample injected after the measurement of calibration standard 50.0 µg/mL sample, (D) plasma specimen of a patient treated levetiracetam (2000 mg/day). LEV, 45.03 µg/ml. The chromatograms monitor LEV at m/z=171.20>126.00 and IS m/z=307.0>220.00.
Figure 3: LC-MS/MS chromatograms of (A) a blank human saliva samples, (B) a saliva sample at the LLOQ level, (C) extracted blank saliva sample injected after the measurement of calibration standard 30.0 µg/mL sample, (D) saliva specimen of a patient treated levetiracetam (500 mg/day); LEV, 14.54 µg/ml. The chromatograms monitor LEV at \( m/z = 171.20 > 126.00 \) and IS \( m/z = 307.0 > 220.00 \).
The calibration curves of LEV in both plasma and saliva were linear over the entire concentration ranges. After comparing the different weighting models, a regression equation with a weighting factor of 1/x² of the drug to the IS concentration was found to produce the best fit for the concentration-detector response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥ 0.99. Calibration curve plot (Figure 4) is shown below. The calculated LLOQs were 1.0 µg/mL for LEV in plasma and 0.5 µg/mL in saliva; and thus below the concentration of the lowest calibration standard. The LLOQ in the plasma was <8.58% RSD and of 3.8% accuracy, respectively, and in the saliva was <2.57% RSD and of 3.4% accuracy, respectively. This assay proved to be sufficiently sensitive for the pharmacokinetic analysis of levetiracetam in plasma and saliva. The chromatograms LLOQ of plasma (Figure 2) and saliva (Figure 3) are shown.

Carry-over and dilution integrity: No peaks in the retention time of LEV and IS were observed in the chromatograms of immediately injected blank plasma samples following the injection of ULOQ or HQC samples, which demonstrated that the carry-over effect could be negligible during the sample analysis. The upper concentration limits can be extended to 100.0 µg/mL for levetiracetam by ½ dilution with screened human blank plasma and saliva. The mean back calculated concentrations for ½ dilution samples were within 91.16-105.93% for plasma and 95.25-101.03% for saliva of their nominal concentrations. The %CV for ½ dilution samples were 4.97% for plasma and 3.48% for saliva and the nominal percentages for ½ dilution samples were 89.55% for plasma and 97.35% for saliva.

Accuracy and precision: Accuracy and precision data for intra-day and inter-day plasma and saliva samples for levetiracetam are summarized in Table 3 and Table 4. The intra-day accuracies varied from 103.8 and 107.4% for plasma and from 101.92 and 104.83% for saliva with a precision of 1.82-8.58% and of 1.01-2.89% for plasma and saliva respectively. Furthermore, the inter-day accuracies varied from 101.2 and 106.0% for plasma and from 100.68 and 104.82% for saliva, with a precision of 1.80-4.79% and of 1.90-3.66% for plasma and saliva, respectively. The assay values on both the occasions (intra-day and inter-day) were found to be within the accepted variable limits.

Stability: The stability of levetiracetam in matrix samples were evaluated under the conditions described in Table 5 and Table 6. The samples were stable under the storage conditions with acceptable accuracy and precision. The results of the tested samples were within the acceptance criteria.

Application of Method

<table>
<thead>
<tr>
<th>S No</th>
<th>Plasma</th>
<th>Saliva</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MF</td>
<td>IS MF</td>
</tr>
<tr>
<td></td>
<td>QC low</td>
<td>QC high</td>
</tr>
<tr>
<td>1.</td>
<td>0.974</td>
<td>0.957</td>
</tr>
<tr>
<td>2.</td>
<td>0.983</td>
<td>0.966</td>
</tr>
<tr>
<td>3.</td>
<td>1.146</td>
<td>0.952</td>
</tr>
<tr>
<td>4.</td>
<td>1.053</td>
<td>0.990</td>
</tr>
<tr>
<td>5.</td>
<td>1.079</td>
<td>0.955</td>
</tr>
<tr>
<td>6.</td>
<td>1.136</td>
<td>0.958</td>
</tr>
</tbody>
</table>

Mean: 1.062  SD: 0.073  CV%: 6.91  1.062  0.073  6.91

Table 2: Matrix effects in human plasma and saliva represented as MF for levetiracetam and MF for IS.

Figure 4: Linearity calibration curve of levetiracetam in plasma (A) and saliva (B).

Recovery
The results of the recovery tests for the three levels tested are shown in Table 1 for plasma and saliva. The extraction recoveries of LEV ranged from 79.87 to 87.87% for plasma and from 92.83 to 94.50% for saliva. The extraction recoveries of IS ranged from 82.78 to 87.23% for plasma and from 98.81 to 101.15% for saliva. The routine standard curve was calculated for each plasma and saliva sample. The extraction recoveries were consistent over the entire concentration range and comparable to the respective internal standard (Table 1).

Method validation
Selectivity: In the chromatograms from six lots of blank plasma samples or saliva samples, no interference peaks from endogenous substances were observed at the retention time of Levetiracetam (1.079 min) and IS (1.072 min) under the established chromatography condition, as shown in the representative chromatogram of a blank plasma sample in Figure 2 and a blank saliva sample in Figure 3. The LOD was determined by LOD=3 × (SD/S), where SD is the standard deviation of the response of the blank and S is the slope of calibration curve. The LOD values for levetiracetam were found to be 0.20 µg/ml for plasma and 0.11 µg/ml for saliva.

Matrix effect: The matrix factor of LEV (LQC) ranged from 0.974-1.146 for plasma and from 0.967-1.130 for saliva. The matrix factor of LEV (HQC) ranged from 0.952-0.990 for plasma and from 0.990-1.049 for saliva. These results were within the acceptance criteria of 0.80-1.20 indicating that no undetected co-eluting compounds that could influence the ionization of the analytes (Table 2).

Limit of quantification and linearity: The calibration curves of respectively. The total run time was 2.0 min, which has advantages over other methods described in the literature [8,12].
The validated method has been successfully used to analyse levetiracetam concentrations in four patients (Table 7). Patients gave their written informed consent to participate and the study procedures were approved by the local ethics committee. Blood samples were withdrawn in the morning, before the drug intake. Saliva samples were collected by the same physician within 5 to 10 minutes after blood sampling.

Conclusion

In summary, a selective and sensitive LC-MS/MS method for determination of levetiracetam in human plasma or saliva was successfully developed and validated. A simple extraction procedure and isocratic chromatography conditions provides an assay well suited for real-time analyses. The method provide a short analysis, superior sensitivity with the lower limit of quantitation as low as 1.0 µg/mL for levetiracetam in plasma and 0.5 µg/mL for drug in saliva. The assay requires only 20 µL plasma or saliva volume and is easy to perform with minimal sample preparation. The method adhered to the regulatory requirements for selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect and stability. From the results of the validation parameters, we can conclude that the developer method can be useful for Bioequivalence studies and routine therapeutic drug monitoring with desired precision and accuracy.

Acknowledgements

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References

drugs by use of saliva. Ther Drug Monit 35: 4-29.


