A Simple HPLC-UV Method For Simultaneous Determination of Levetiracetam and Carbamazepine

Levetirasetam ve Karbamazepin’in Birlikte Analizi İçin Basit Bir HPLC-UV Metodu

Busra KANDILLI¹
Afife Busra UGUR¹
Meltem CETIN*²
Fatma DEMIRKAYA MILOGLU²

¹Ataturk University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Erzurum
²Ataturk University, Faculty of Pharmacy, Department of Analytical Chemistry, Erzurum

*Corresponding Author:
Meltem CETIN
Ataturk University, Faculty of Pharmacy, Department of Pharmaceutical Technology, Erzurum
E-mail: melcetin@atauni.edu.tr; melcetin@hotmail.com
Tel: +90 442 231 5236

ABSTRACT
A combination of levetiracetam (LEV) and carbamazepine (CBZ) may be used to obtain synergistic effect in treatment of epilepsy. The aim of the study is to develop and validate a simple reversed phase (RP) HPLC-UV method to determine simultaneously LEV and CBZ in pure form and in different pharmaceutical formulations. Separation was achieved on a C18 column (5 μm, 4.6mm×250mm) using a mobile phase consisting of methanol and ultrapure water (45:55, v/v) at a flow rate of 1.0 mL/min at 230 nm. Validation parameters (specificity, linearity, accuracy, precision, LOD and LOQ) were studied according to the relevant ICH Guideline. The calibration curves were linear over a concentration range of 0.5-32 μg/mL for both LEV and CBZ. The intra- and inter-day accuracy values (as relative error; RE) were less than ±2% and also the intra- and inter-day precision values (as the percent relative standard deviation; RSD%) were less than 2%. According to relevant guidelines, the accuracy and precision of the developed method is suitable. LOD and LOQ values were found to be 0.036 and 0.110 μg/mL for LEV and 0.026 and 0.078 μg/mL for CBZ, respectively. The simple, specific, accurate, precise and sensitive analytical method developed could be useful for simultaneous determination of LEV and CBZ.

Keywords: Carbamazepine, HPLC-UV method, Levetiracetam, Validation.

ÖZET
Epilepsi tedavisinde sinerjik etki elde etmek için levetirasetam (LEV) ve karbamazepin (CBZ) birarada kullanılabilir. Bu çalışmanın amacı, saf halde ve farklı farmasötik formülasyonlarda LEV ve CBZ’yı eş zamanlı olarak belirilecek üzere basit ters faz (RP) HPLC-UV yönteminin geliştirilmesi ve geçerliliğinin gösterilmesidir. Aşırı, C18 kolonunda (5 μm, 4.6 mm x 250 mm), metanol ve ultra saf sudan (45:55, h/h) oluşan hareketli faz kullanılarak 1.0 mL/dk akış hızında ve 230 nm’de gerçekleştirildi. Geçerlilik parametreleri (özgüllük, doğrulsallık, doğruluk, kesinlik, LOD ve LOQ) ilgili ICH Kılavuzuna göre çalışmıştır Kalibrasyon eğrileri hem LEV hem de CBZ için 0.5-32 μg/mL derişim aralığında doğrusal olarak bulunmuştur. Gün içi ve günler arası doğruluk değerlerinin [bağıl hata (BH) olarak] ±2% den az ve gün içi ve günler arası kesinlik değerlerinin de (% baglı standart sapma; %BSS) %2 den az olduğu saptanmıştır. Iğili kılavuzlara göre, geliştirilen yöntemin doğruluğu ve kesinliği uygundur. LOD ve LOQ değerleri sırasıyla, LEV için 0.036 ve 0.110 μg/mL ve CBZ için sırasıyla 0.026 ve 0.078 μg/mL olarak bulunmuştur. Geliştirilen basit, özgül, doğru, kesin ve hassas analitik yöntemi LEV ve CBZ’in eş zamanlı olarak sağınlaması için faydalı olabilir.

Anahtar Kelimeler: Geçerlilik, HPLC-UV yöntemi, Karbamazepin, Levetirasetam

1. Introduction
Epilepsy is a chronic disease characterized by a defined unprovoked and repeated seizure that occurs suddenly in the cortical neurons, resulting in excessive electrical discharge. During seizures, epilepsy patients...
suffer from loss of consciousness, sense and movement control, autonomic and psychic disturbances [1]. Epilepsy affects about 40 million people worldwide and can be seen at any age and requires long-term treatment. The incidence of epilepsy is higher in developing countries due to limited health services such as malnutrition, birth and head trauma [2,3]. Epileptic seizures are controlled by various methods such as surgical treatment, ketogenic diet, vagal stimulation and most preferred antiepileptic drugs (AEDs) therapy that reduces seizure frequency and severity. The AEDs to be used in treatment (conventional/classical, second generation) should be effective, long-term preservative, well tolerated and increase the quality of life of the patient [2,4]. Appropriate drug therapy should be started with a single drug called monotherapy, considering seizure type and epilepsy syndrome. After failure of monotherapy regimens due to the lack of efficacy, polytherapy as a combination of different AEDs can be considered for the treatment of epilepsy with minimal side effects, synergistic/additive effect of drugs [5,6]. Florek-Luszczki et al. [7] found that a combination of LEV and CBZ showed synergistic/additive effect in mice 6Hz psychomotor seizure models.

LEV, (S)-α-ethyl-2-oxo-pyrrolidine acetamide (Figure 1a), is chemically similar to the prototypical nootropic agent piracetam. LEV is a new generation antiepileptic drug that has been used in recent years and is effective as a monotherapy for epilepsy in the case of partial onset seizures with or without secondary generalisation, or as an adjunctive therapy for tonic-clonic seizures, partial and myoclonic [8, 9]. The mechanism of action of LEV is not fully understood, but it has been reported to act by binding to synaptic vesicle protein SV2A [10]. LEV has high solubility and permeability is quickly and nearly entirely absorbed after oral administration [11].

CBZ, 5-H-dibenze[b,f]azepine-5-carboxamide (Figure 1b), is a tricyclic lipophilic compound that is a first choice antiepileptic drug to control secondarily generalised tonic-clonic seizures and partial seizures [12]. CBZ particularly shows good antiepileptic activity by blocking sodium channels [13]. It has low solubility in water and irregular and often slow oral absorption [14].

Figure 1. Chemical structure of LEV (a) and CBZ (b) [15].

A number of methods have been reported for the determination of only LEV or CBZ [21-23]. HPLC-DAD, UPLC-MS/MS and LC-MS/MS methods were used for the simultaneous determination of LEV and CBZ in blood/human plasma/tissues [21-23]. The aim of the study is to develop and validate a simple and rapid HPLC-UV method to determine simultaneously of LEV and CBZ in pure form and also in different pharmaceutical formulations.

2. Materials and Methods

2.1. Materials

LEV and CBZ were generous gifts from DEV A Holding AŞ (Istanbul, Turkey) and Biofarma İlaç San. Tic. A.Ş. (Istanbul, Turkey), respectively. Methanol (HPLC grade) was purchased from Sigma Aldrich (Germany) and ultra-pure water was obtained from Millipore (Direct-Q® 3UV, USA).

2.2. Methods

2.2.1. Determination of Appropriate UV Wavelength

The solutions of LEV and CBZ in methanol (10 μg/mL) were scanned in the range of 200-600 nm using with a UV spectrophotometer (Beckman Coulter-DU ® 730, USA). Methanol was used as a blank and suitable wavelength was determined.

2.2.2. Chromatographic System and Conditions

An HPLC system (Thermo Finnigan Surveyor, USA) equipped with UV detector and autosampler was employed in this study. Chromatographic software Chromoquest was used for data collection and processing. Separation was achieved on a C18 column (5 μm, 4.6 mm×250 mm, Supercosil LC-18-DB, Supelco) using a mobile phase consisting of methanol and ultrapure water (45:55, v/v), at the same time the temperature of the column was kept constant at 25°C. Flow rate of the mobile phase was set as 1.0 mL/min and UV detection was performed at 230 nm after an injection of 10 μL sample.

2.2.2.1. Preparation of Standard Solutions

The stock solution of LEV and CBZ (50 μg/mL for both drugs) in mobile phase was diluted by using mobile phase to prepare the standard solutions in the concentration range of 0.5-32 μg/mL.

2.2.2.2. Method Validation

The method was validated according to the International Conference on Harmonization (ICH) Q2 (R1) guideline for the accompanying parameters such as specificity, linearity, precision, accuracy, the limit of detection (LOD) and the limit of quantitation (LOQ) [24].
Specificity
Specificity is defined as the ability of the method to discriminate the response of the target compound from the responses of other sample components [25]. The specificity of the method was assessed by examination of the chromatograms to check the absence of apparent shoulders or interfering peaks. The specificity of the method for the determination of LEV and CBZ was established by injecting the mobile phase.

Linearity and Range
For linearity of study, the standard solutions (n=6) in the concentration range of 0.5-32 µg/mL of both LEV and CBZ were prepared. The calibration curves were constructed by plotting the respective peak areas of LEV and CBZ against their concentrations. The linearity of the method was evaluated by using linear regression analysis and regression equations, the values of the slope, the intercept and the coefficient of determination ($r^2$) were obtained.

Accuracy
The accuracy of the method expresses the closeness of the value found (the test results obtained by the analytical method) to the true value [26]. Intra- and inter-day accuracy (as relative error, RE) was determined by the assay of six replicate of freshly prepared standard solutions (1, 8 and 24 µg/mL) for both LEV and CBZ on same day and three different days, respectively.

Precision
The precision of the method is defined as the closeness of agreement between a series of measurements under the prescribed conditions [27]. The variability from repeated analyses over a short interval of time under the same operating conditions within a laboratory is defined as intra-day precision (repeatability). Validation precision should also include intermediate precision which expresses within-laboratories variations (different days, different equipment, different analysts, etc) [24]. Intra- and inter-day precision was determined by the assay of six replicate of freshly prepared standard solutions (1, 8 and 24 µg/mL) for both LEV and CBZ on same day and three sequential days, respectively. Intra- and inter-day percentage relative standard deviation (RSD%) values were calculated.

LOD and LOQ
LOD is defined as the lowest concentration of analyte in a sample which could be detected but not necessarily quantified. LOQ is expressed as the lowest concentration of analyte in a sample which can be measured with suitable precision and accuracy [24]. In this study, LOD and LOQ were determined according to ICH Q2 (R1) recommendations and calculated using the following equations [24]:

$$\text{LOD} = 3.3 \times \frac{\sigma}{S}$$

$$\text{LOQ} = 10 \times \frac{\sigma}{S}$$

σ is the standard deviation of the intercept and S is the slope of the calibration curve.

3. Results and Discussion
In this study, a simple and sensitive HPLC-UV method was developed and validated for quantitative and simultaneous determination of LEV and CBZ. The chromatographic conditions were determined to provide a good performance of the assay. LEV is a white/off-white crystalline powder with a bitter taste and a faint odor and it is very soluble in water and freely soluble in methanol. CBZ is a white/off-white powder, which is practically insoluble in water and soluble in alcohol.

Initially, the obtained UV spectra of the solutions of LEV (10 µg/mL) and CBZ in methanol (10 µg/mL) showed maximum absorbance at 201 nm (for LEV) and 285 nm (for CBZ) (Figure 2). The wavelength for the simultaneous analysis of LEV and CBZ was selected as 230 nm according to the previous study published by Ibrahim et al [22].

![Figure 2. UV spectrums of LEV (a) and CBZ (b).](image-url)
After preliminary experiments, the mixture of methanol and ultrapure water (45:55, v/v) was selected as mobile phase based on the solubility of LEV and CBZ, ease of preparation, run time and peak parameters. Isocratic chromatographic separation was achieved on a C18 column (5 μm, 4.6 mm×250 mm), at a flow rate of 1.0 mL/min and UV detection at 230 nm. A chromatogram of LEV and CBZ under the above mentioned chromatographic conditions was given in Figure 3. The total run time for the assay was less than 15 min with a clear separation. The retention time of LEV and CBZ were recorded as 3.4 min and 12.4 min, respectively and were indicated a good base line.

Ibrahim et al. [22] developed a RP-HPLC-DAD method for simultaneous determination of several antiepileptic drugs including LEV and CBZ in human plasma. They used a C18 column and the mixture of methanol, acetonitrile and water (30:10:60 v/v/v) as mobile phase for separation. The active substances are detected at 230 nm. The retention times of LEV and CBZ in the different samples of human plasma were found as about 3.36 min and 21.89 min, respectively [22]. In our study, the developed method is sufficiently rapid for determination of LEV and especially CBZ.

The analytical method was validated with respect to specificity, linearity, precision, accuracy, LOD and LOQ. The specificity describes the capacity of the assay to measure the active substances in the presence of excipients or matrix components [24]. The parameter was determined by comparing the chromatogram of the standard solutions containing LEV and CBZ and only mobile phase. The chromatogram of standard solutions containing LEV and CBZ (Figure 3a) show two peaks in the retention time about 3.4 min and 12.4 min, respectively. In the chromatogram of mobile phase, no peak is observed in the regions of the peaks of LEV and CBZ (Figure 3b). The peaks of LEV and CBZ are well resolved, indicating the high specificity of the developed HPLC-UV method.

The analytical curves for LEV and CBZ were constructed by plotting the area of the peak (mAu) versus the concentration of active substance (μg/mL). The regression equations of LEV and CBZ were $y=2039.1x-120.54$ and $y=38499x-499.58$, respectively (Figure 4). A good linear relationship for both LEV and CBZ was established between their peak areas and their concentrations in the concentration range (0.5-32 μg/mL) with the coefficients of determination were 0.99996 for LEV and 0.99994 for CBZ.

![Figure 3](image-url)  
**Figure 3.** HPLC chromatograms of (a) LEV (0.5-32 μg/mL) and CBZ (0.5-32 μg/mL), (b) mobile phase.
The accuracy and precision were evaluated in terms of RE and RSD%, respectively. The intra-day and inter-day accuracy of the HPLC method for LEV (1, 8 and 24 µg/mL) and CBZ (1, 8 and 24 µg/mL) were appropriate (RE is <±2% for the in vitro conditions [16]). The calculated RE (%) for both LEV and CBZ were in the range of -0.109-1.785% and 0.015-1.774%, respectively (Table 1). The intra-day and intra-day precisions (RSD%) for LEV (1, 8 and 24 µg/mL) and CBZ (1, 8 and 24 µg/mL) were in the range 0.135-1.704% and 0.261-1.341%, respectively (RSD% is < 2% for the in vitro conditions [16]), the results for precisions were given in Table 1. Also, the results of injection repeatability given in Table 2 were shown that the ranges of RSD% calculated for both LEV and CBZ were 0.368-0.887% and 0.076-0.420%, respectively. The values mean good accuracy and precision, therefore the method is adequate for routine simultaneous quantification of LEV and CBZ.

LOD and LOQ were determined by injecting lower concentrations of the standard solutions of analytes using the optimized chromatographic conditions. The LOD and LOQ values were found to be 0.036 µg/mL and 0.110 µg/mL for LEV and also 0.026 µg/mL and 0.078 µg/mL for CBZ, respectively.

![Figure 4. Calibration curves of LEV (a) and CBZ (b).](image)

Table 1. The results of intra- and inter-day accuracy and precision.

<table>
<thead>
<tr>
<th>Added Concentration (µg/mL)</th>
<th>Found Concentration (µg/mL; X±SD)</th>
<th>Accuracy (RE, %)</th>
<th>Precision (RSD%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LEV</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.015 ± 0.015</td>
<td>1.518</td>
<td>1.491</td>
</tr>
<tr>
<td>8</td>
<td>8.097 ± 0.138</td>
<td>1.192</td>
<td>1.704</td>
</tr>
<tr>
<td>24</td>
<td>24.100 ± 0.198</td>
<td>0.415</td>
<td>0.822</td>
</tr>
<tr>
<td><strong>INTER-DAY</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.018 ± 0.014</td>
<td>1.774</td>
<td>1.341</td>
</tr>
<tr>
<td>8</td>
<td>8.061 ± 0.021</td>
<td>0.757</td>
<td>0.261</td>
</tr>
<tr>
<td>24</td>
<td>24.004 ± 0.071</td>
<td>0.015</td>
<td>0.297</td>
</tr>
<tr>
<td><strong>CBZ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.018 ± 0.001</td>
<td>1.785</td>
<td>0.135</td>
</tr>
<tr>
<td>8</td>
<td>7.991 ± 0.073</td>
<td>-0.109</td>
<td>0.910</td>
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<tr>
<td>24</td>
<td>24.086 ± 0.209</td>
<td>0.359</td>
<td>0.868</td>
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<tr>
<td><strong>INTER-DAY</strong></td>
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<tr>
<td>1</td>
<td>1.011 ± 0.005</td>
<td>1.061</td>
<td>0.466</td>
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<tr>
<td>8</td>
<td>8.035 ± 0.083</td>
<td>0.436</td>
<td>1.032</td>
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<tr>
<td>24</td>
<td>24.038 ± 0.140</td>
<td>0.156</td>
<td>0.580</td>
</tr>
</tbody>
</table>

X: Mean; SD: Standard Deviation
Table 2. The results of injection repeatability of both LEV and CBZ.

<table>
<thead>
<tr>
<th>Added Concentration (μg/mL)</th>
<th>Found Concentration (μg/mL; X±SD)</th>
<th>RSD%</th>
</tr>
</thead>
<tbody>
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<td>LEV</td>
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<tr>
<td>1</td>
<td>1.009 ± 0.009</td>
<td>0.887</td>
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<tr>
<td>8</td>
<td>8.007 ± 0.062</td>
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<tr>
<td>24</td>
<td>24.107 ± 0.089</td>
<td>0.368</td>
</tr>
<tr>
<td>CBZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.006 ± 0.004</td>
<td>0.420</td>
</tr>
<tr>
<td>8</td>
<td>7.991 ± 0.013</td>
<td>0.158</td>
</tr>
<tr>
<td>24</td>
<td>24.018 ± 0.018</td>
<td>0.076</td>
</tr>
</tbody>
</table>

X: Mean; SD: Standard Deviation

4. Conclusion

The RP-HPLC-UV method was developed and validated according to the relevant ICH guideline for simultaneous determination of LEV and CBZ. The proposed method had a good agreement with all verification parameters and was found to be specific, linear, accurate, precise and sensitive for a reliable quantitative and simultaneous determination of LEV and CBZ. The primary advantage of the identified method is to be analyzed the amount of LEV and CBZ on a single chromatographic system. Hence, this method may be used for routine analysis of LEV and CBZ in pure form and in different pharmaceutical formulations.

5. References

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