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Full Length Research Paper

Simple and rapid ultra high performance liquid chromatographic (UHPLC) method for the determination of levetiracetam in human serum: Application to a bioequivalence study

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A rapid and valid method is reported for the determination of levetiracetam (LEV) in human plasma with ultra high performance liquid chromatography (UHPLC) and ultraviolet (UV) detection. To 100 µl serum 50 µl lamivudine (IS, 10 µg/ml) was added as an internal standard and the mixture subjected to liquid extraction using 1 ml ethylacetate. A mixture of acetonitril and distilled water (80:20) was used as mobile phase and the analysis performed on a Blue Orchid C_{18} (1.8 µm, 50 × 2 mm) column using diode array detector operated at 205 nm. The drug and IS were eluted at 0.8 and 1.2 min, respectively. The retention times for LEV and IS were 0.8 and 1.2, respectively. The method was rapid with analytical run time of 1.2 min and sensitive to the measurement of LEV in human serum following single dose administration of the drug with limit of detection (LOD) and limit of quantification (LOQ) at 0.02 and 0.05 µg/ml, respectively. Calibration curves (mean correlation coefficient = 0.9988) were linear over the concentration ranges of 0.05 to 1 µg/ml. The valid method was used in a randomized crossover bioequivalence study of two different LEV preparations in 24 healthy volunteers.

Key words: Levetiracetam, ultra high performance liquid chromatography (UHPLC), diode array detector, bioequivalence study.

INTRODUCTION

Levetiracetam ((S)-α -ethyl-2-oxo-1-pyrrolidine acetamide, LEV) (Figure 1) is one of the newest antiepileptic agents, marketed worldwide only since 2000 (Abou-Khalil, 2008). LEV has been approved as a monotherapy treatment for epilepsy in partial seizures, or as an adjunctive therapy for partial myoclonic and tonic-clonic seizures (Gambardella et al., 2008; Berkovic et al., 2007; Ben-Menachem and Falter, 2000), and also, it is used in the treatment neurologic diseases such as anxiety disorder, Tourette syndrome and autism (Farooq et al., 2009). The exact mechanism by which LEV acts to treat epilepsy is unknown. However, the drug binds to a synaptic vesicle protein, SV2A which is believed to inhibition nerve conduction across synapses (Lynch et al., 2004). In the pharmacokinetic properties of LEV, it is rapidly and almost completely absorbed after oral intake, with peak plasma concentrations approximately 1 h after oral administration and it has linear behavior. LEV protein binding is at less than 10%, and plasma half-life is 7 ± 1 h in adults (Patsalos, 2003, 2004). Quantitation of LEV levels in patient plasma is very important, because there is need to adjust the dose for patient to decrease adverse effects (Brodtkorb et al., 2004). Thus, a simple and rapid method is needed for therapeutic drug monitoring (TDM) and analysis of this drug in pharmacokinetic study after

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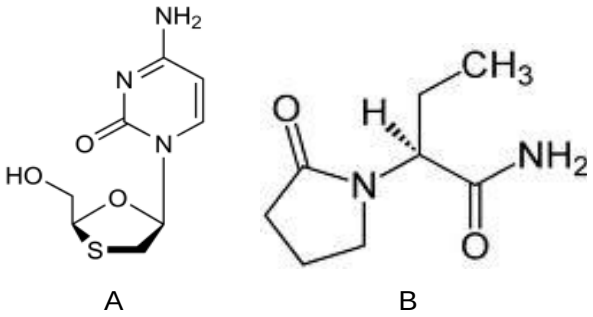

Figure 1. Chemical structure of (A) Lamivudine and, (B) Levetiracetam.

single oral administration. Several analytical methods have been reported for the analysis of LEV in human serum, and among them are gas chromatography (GC, with nitrogen-phosphorus and mass detector) (Vermeij and Edelbroek., 1994; Isoherranen et al., 2000), LC tandem mass (Matar, 2008; Mendu and Soldin, 2010), HPLC (Martens-Lobenhoffer and Bode-B¨oger, 2005; Contin et al., 2008), capillary electrophoresis (Shihabi et al., 2003), and electrochemical method (Alonso-Lomillo et al., 2009). These methods have several problems such as low sensitivity, time consuming, need large volume of sample and some of them require solid phase extraction. One method is reported for analysis of LEV using ultra high performance liquid chromatography (UHPLC) tandem mass spectrometry. The limit of quantification (LOQ) and limit of detection (LOD) used in this study were 0.15 and 0.06 µg/ml, respectively and they showed low sensitivity as compared to our assay. Retention times for LEV and IS were 2.1 and 2.2 min more than that of this study; also, this method is cumbersome and expensive (Blonk et al., 2010). In this study, we apply a new, simple, fast and valid method for the determination of LEV in human plasma with UHPLC-UV detection. This method was applied for quantification of the drug in a bioequivalence study following oral administration of two different LEV preparations in 24 healthy volunteers.

EXPERIMENTAL

Chemicals

LEV was from Bakhtar Bioshimi (Kermanshah, Iran) and lamivudine (Figure 1) as an IS was from Sigma (Sigma, St .Louis, MO, USA). HPLC grade acetonitril, methanol, and ethylacetate were purchased from Merck (Darmstand, Germany). All reagents were of the maximum available purity and were used without further purification. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

Standard solutions

The stock solution of LEV (1000 µg/ml) and IS (10 µg/ml) were prepared in methanol. All solutions were stored at 4°C and were stable for at least 4 weeks. Stock solutions were prepared freshly before the analysis.

Instrumentation

The UHPLC system used consisted of two pumps of platin blue P-1 solvent delivery system, a diode array detector (PDA-1) operated at 205 nm, a column temperature manager (T-1) and analytical column (Blue Orchid C_{18}, 1.8 µm, 50 × 2 mm), all from Knauer, Berlin, Germany. A mixture of acetonitril and distilled water (20:80) was used as the mobile phase. The column oven temperature was set at 40°C and the mobile phase was filtered and pumped at a flow rate of 0.2 ml/min.

Extraction procedure

Serum samples were stored at −40°C until assay. Frozen serum samples were thawed in water at 37°C. Liquids of blank, calibration standard or unknown human serum samples (100 µl) were pipetted in to microtube, containing 50 µl of working internal standard solution. The samples were extracted with 1 ml of ethylacetate. After vortex mixing for 30 s and 3 min centrifugation (14000 rpm), the supernatant was transferred into glass tube. Then, organic phase was evaporated in 60°C, and the residual dissolved in 50 µl methanol and 3 µl was injected onto UHPLC system.

Preparation of calibration curve standards

Samples for calibration curves were prepared within the concentration range of 0.05 to 1 µg/ml. In disposable glass tubes (100 × 16 mm), after evaporation of 100 µl from each working solutions of the drug, under a gentle stream of nitrogen at 60°C, the residues were reconstituted in 100 µl of drug-free human serum and was mixed for 10 s on a vortex mixer and the samples were subjected to extraction, and analysis as explained earlier.

Validation of the method

Assay linearity was studied with six calibration standards (0.05, 0.1, 0.25, 0.5, 0.75, and 1 µg/ml) in duplicate using blank serum samples obtained from healthy volunteers. Calibration curves (weighted regression line) were obtained by linear least-squares regression analysis of plots of peak-area ratio of LEV to IS against drug concentrations. Quality control samples used in the method validation were prepared with the drug working solutions to make low (0.05 µg/ml), medium (0.25 µg/ml), and high (1 µg/ml) concentrations. Within and between day variations were determined by repeated analysis (n = 6) of different concentrations of the drug in a single analytical run and in 10 analytical runs performed on different days, respectively, using the same stock solutions and serum batches. The specificity of the method was examined by the presence of disturbing endogenous peaks in 24 human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the IS. The absolute recoveries of LEV at the aforementioned concentrations as well as the IS at applied concentration were calculated in replicates (n = 5) by comparing the respective peak-areas obtained of the extracted samples from serum, with those that obtained the same amounts of un-extracted solutions in methanol. The LOD and quantification LOQ were defined as the concentration of the drug giving a signal-to-noise ratio of 3:1 and 10:1, respectively, and the stability solution of LEV and the IS was studied over a period of 4 weeks by comparing the peak-areas at different times. Stability of the drug in serum samples

was examined by comparing the determined concentration in different times up to 30 days maintenance of the samples at −40°C and following three thaw-freeze cycles.

Application of the method

The present method was applied in a randomized crossover bioequivalence assay of two different LEV preparations. The study protocol was approved by university medical ethics committee. Twenty-four male healthy volunteers aged 27.2 ± 3.1 years and weighing 67.7 ± 8.3 kg with normal biochemical parameters were enrolled in this study. All the subjects were divided in two groups and they received a single oral dose of 500 mg LEV from either Bakhtar Bioshimi (Kermanshah, Iran) or USB pharmaceutical companies. All the subjects were asked to refrain from food or water consumption for 3 h after drug administration, and on two working days, they were separated by a wash-out period of 3 weeks. Blood sampling were carried out at suitable intervals up to 24 h and pharmacokinetic parameters including maximum concentration (C_{max}), area under the concentration time curve from zero to the time of last sampling (AUC_{0-t}), and area under the concentration time curve from zero to infinity ($AUC_{0-\infty}$) were compared. To establish bioequivalence, 90% confidence interval for AUC and C_{max} within ranges of 80 to 120% was calculated.

RESULTS

Specificity and selectivity

The validated method was demonstrated excellent chromatographic specificity with no endogenous serum interference at the retention times of LEV and IS. Figures 2A and 2B Representative chromatograms were for human blank serum and spiked with LEV (0.05 µg/ml) and the IS, respectively. No endogenous peaks from serum were found to interfere with the elution of the drug or IS. LEV and the IS were well resolved with good symmetry with respective retention times of 0.8 and 1.2 min. Figure 2C shows the chromatogram of serum sample obtained at 3 h after a single oral dose of 500 mg LEV from a healthy volunteer.

Recovery, precision, accuracy and stability

The recoveries of LEV and IS from serum were determined by extraction of spiked serum samples when compared with peak areas obtained after the same amounts of unextracted LEV solutions in methanol. The mean recoveries were found to be 98 ± 9% for LEV and 93 ± 8% for the IS. The intra-day and inter-day precision and accuracy of the assay were examined by analyzing replicate serum samples spiked with different amounts of the drug within calibration curve range on the same day and on 10 different days. The intra-day and inter-days accuracy and precision values of the assay method are presented in Table 1. The coefficient of variation values of intra-day and inter-day were less than 8 and 7.1%, respectively, whereas the accuracy of the method was 97.5 to 103.5% (intra-day) and 97.2 to 103.2% (inter-day).

Stock solutions of LEV and SI were stable at least for 60 days when stored at 4°C. Extracted serum was found to be stable for at least 24 h if the samples were kept at room temperature (20 to 30°C). The concentrations of LEV in serum stored at -80°C for 60 days and following three freeze-thaw cycles were found to be 100 ± 2% from the initial values.

Sensitivity and linearity

The detection limit for LEV was approximately 0.02 µg/ml at a signal-to-noise ratio of 3:1 and the quantification limit corresponding with a coefficient of variation of less than 20% was 0.05 µg/ml using 100 µl serum sample and 3 µl injection. The standard calibration curves were linear over the concentration ranges of 0.05 to 1 µg/ml using line-fit plot in regression analysis with a coefficient of 0.9988 and regression equation of $y = 0.1123x - 0.7353$. Intra and inter-day reproducibility for calibration curves were determined on the same day in replicate (n = 6) and on different days (n = 10), respectively, using same pooled serum sample.

Applied method

This method has been used for analysis of the drug in human serum following single dose administration of 500 mg of two LEV preparations in 24 healthy volunteers. The mean serum LEV concentrations versus time curve of two formulations are as shown in Figure 3. A Cmax of 0.341 ± 0.011 µg/ml was reached at 4.9 ± 1.1 h after administration, AUC0−t and AUC0−∞ were 3.997 ± 0.22 and 6.029 ± 0.36 µg/ml, respectively, and the drug was eliminated with terminal half life of 14.7 ± 5 h.

Conclusion

Conclusively, a very rapid, simple, and sensitive method has been described in the present paper.

In this method which has been demonstrated to be suitable for use in pharmacokinetic studies of LEV when compared with previously published method, less time is needed for analysis of the drug. In this study, simple extraction procedure and inexpensive method was used

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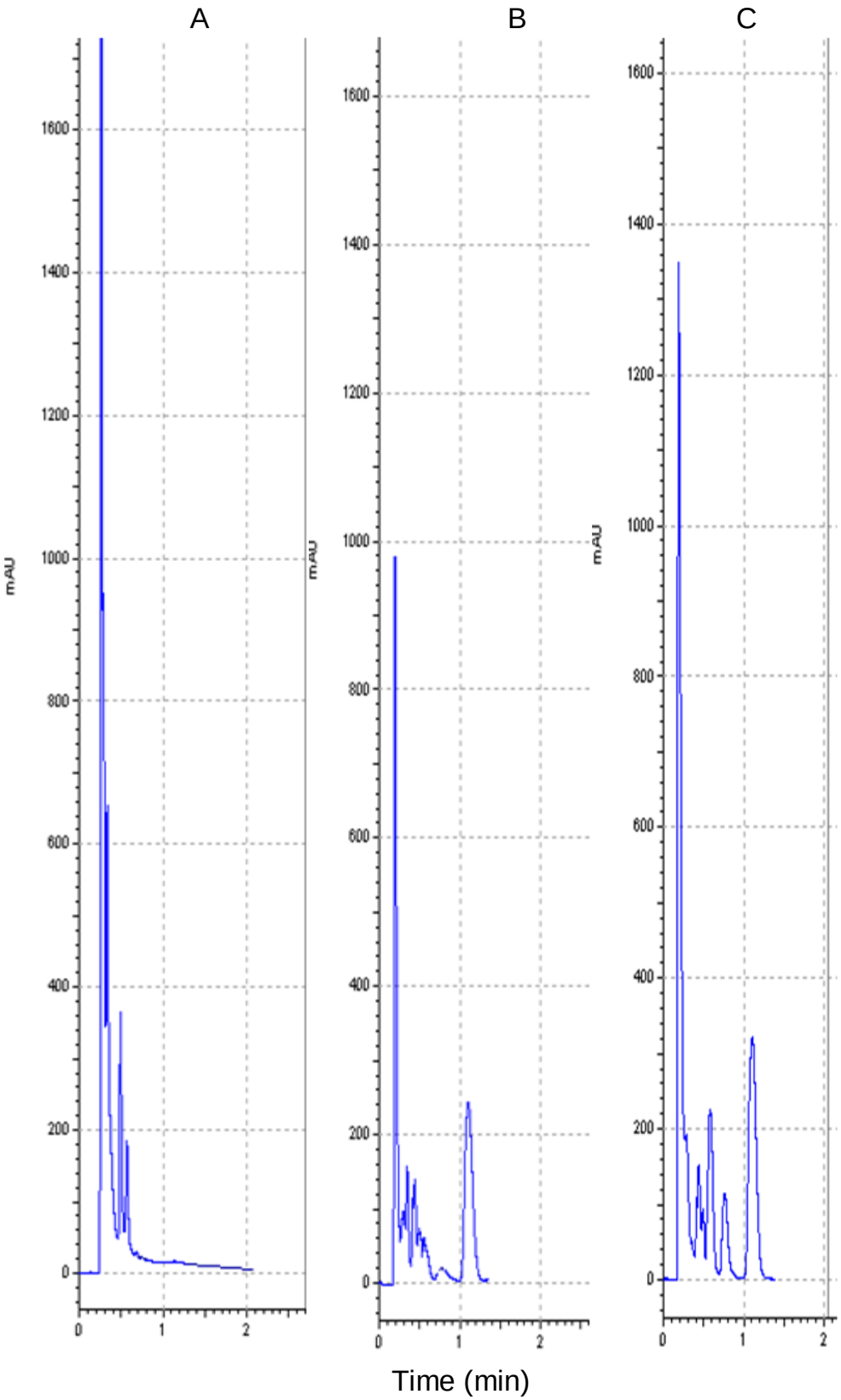

Figure 2. Typical chromatograms obtained from an extract of (A) human blank serum; (B) human blank serum spiked with 0.05 µg/ml LEV and Lamivudine as IS; (C) serum samples obtained at 3 h after a single oral dose of 500 mg LEV from a healthy volunteer. Peaks eluted at 0.8 and 1.2 min correspond to LEV and IS, respectively.

Table 1. Inter and intra-day precision and accuracy for determination of LEV in human serum by the UHPLC method.

Known concentration (µg/mL)	Concentration found (mean +/-S.D)	Coefficient of variation (%)	Accuracy (%mean deviation)
Inter-day (n=10)			
0.05	0.051± 0.003	7.1	3.5
0.25	0.255 ± 0.022	4.0	2.5
1	1.021 ± 0.25	1.5	1.7
Intra-day (n=6)			
0.05	0.051± 0.004	8.0	3.2
0.25	0.257 ± 0.028	4.1	2.8
1	1.018 ± 0.31	2.6	1.6

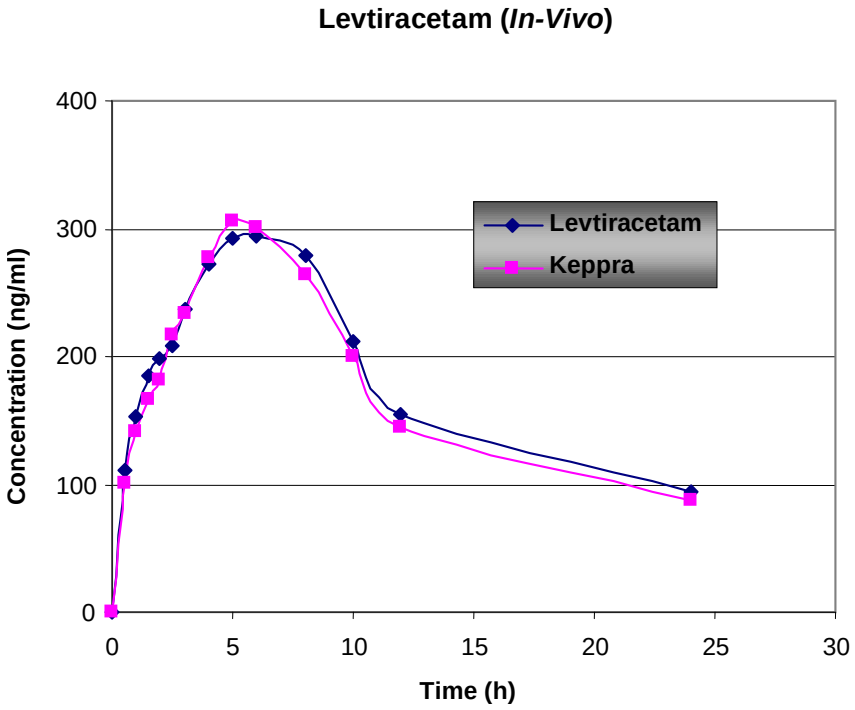

Figure 3. Mean serum concentrations versus time profiles of LEV for two different preparations in 24 human volunteers after administration of a single 500 mg oral dose.

REFERENCES

Abou-Khalil B (2008). Levetiracetam in the treatment of epilepsy. Neuropsychiatr. Dis. Treat 4:507–523.

Alonso-Lomillo MA, Domínguez-Renedo O, Matos P, Arcos-Martínez MJ (2009). Electrochemical determination of Levetiracetam by screen-printed based biosensors. Biol. Elect. Chem. 74:306–309.

Ben-Menachem E, Falter U (2000). Efficacy and tolerability of Levetiracetam 3000 mg/d inn patients with refractory partial onset seizures: a multi center, double-blind responder-selected study evaluating monotherapy. Epilepsy 41:1276–1283.

Berkovic SF, Knowlton RC, Leroy RF, Schiemann J, Falter U (2007). Placebo-controlled study of Levetiracetam in idiopathic generalized epilepsy. Neurology 69:1751–60.

Blonk M, van der Nagel BC, Smit LS, Mathot RA (2010). Quantification of levetiracetam in plasma of neonates by ultra performance liquid chromatography-tandem mass spectrometry J. Chromatogr. B. 878:675–681.

Brodtkorb E, Klees TM, Nakken KO, Lossius R, Johannessen SI (2004). Levetiracetam in adult patients with and without learning disability: focus on behavioral adverse effects. Epilepsy Behav. 5:31–5.

Contin M, Mohamed S, Albani F, Riva R, Brauzzi A (2008). Simple and validated HPLC-UV analysis of levetiracetam in deproteinized plasma of patients with epilepsy. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 873:129–132.

Farooq MU, Bhatt A, Majid A, Gupta R, Khasnis A, Kassab MY (2009). Levetiracetam for managing neurologic and psychiatric disorders". Am. J. Health Syst. Pharm. 66(6):541-561.

Gambardella A, Labate A, Colosimo E, Ambrosio R, Quattrone A (2008). Monotherapy for partial epilepsy: focus on levetiracetam. Neuropsychiatr. Dis. Treat 4(1):33–38.
Isoherranen NM, Roeder S, Soback B, Yagen V, Bialer M (2000). Enantioselective analysis of levetiracetam and its enantiomer R-alpha-ethyl-2-oxo-pyrrolidine acetamide using gas chromatography and ion trap mass spectrometric detection. J. Chromatogr. B. 745:325–332.
Matar K (2008). Quantification of levetiracetam in human plasma by liquid chromatography-tandem mass spectrometry: application to therapeutic monitoring. J. Pharm. Biomed. Anal. 48:822–828.
Lynch BA, Lambeng N, Nocka K (2004). The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug Levetiracetam. Proc. Nat. Acad. Sci. USA. 101(26):9861–9866.
Martens-Lobenhoffer J, Bode-B¨oger SM (2005). Determination of Levetiracetam in human plasma with minimal sample pretreatment. J. Chromatogr. B. 819:197–200.
Mendu DR, Soldin SJ (2010). Simultaneous determination of Levetiracetam and its acid metabolite (ucb L057) in serum/plasma by liquid chromatography tandem mass spectrometry. Clin. Biochem. 43:485–489.
Patsalos PN (2003). The pharmacokinetic characteristics of Levetiracetam Methods Find Exp. Clin. Pharmacol. 25(2):123.
Patsalos PN (2004). Clinical pharmacokinetics of Levetiracetam. Clin. Pharmacokinet. 43:707–724.
Shihabi ZK, Oles K, Hinsdale M (2003) Analysis of the antiepileptic drug keppra by capillary electrophoresis. J. Chromatogr. A. 1004:9-12.
Vermeij TA, Edelbroek PM (1994). High-performance liquid chromatographic and megabore gas-liquid chromatographic determination of Levetiracetam (ucb L059) in human serum after solid-phase extraction. J. Chromatogr. B. Biomed. Appl. 662:134–139.