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Article

Determination of ng/mL Levetiracetam using Ultra-High-Performance Liquid Chromatography–Photodiode Absorbance

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This paper demonstrates the analysis of levetiracetam, a new chiral antiepileptic drug, at ng/mL levels using an ultra-high-performance liquid chromatography (UHPLC)–photodiode absorbance (PDA) method. Three different sample preparation methods, liquid–liquid extraction with Extrelut, solid phase extraction (SPE) with Oasis HLB and Oasis MAX SPE cartridges, and protein precipitation with organic solvents were carried out. The last preparatory method is the simplest and provides the best recoveries: between 97.1% and 100.4% with RSD value below 5%. The column for separation is BEH C18 column (1.7 μm particle size and 100 × 2.1 mm i.d.) and acetonitrile-phosphate buffer (pH = 6.6; 0.01 M) (10/90 v/v) is the mobile phase. The results obtained are compared to analysis conducted by the HPLC method. The UHPLC method was validated in the range of 2–100 μg/mL levetiracetam concentration (R^2 = 0.9997). LOD and LOQ are 10 ng/mL and 33 ng/mL, respectively. The developed UHPLC method was applied to plasma samples of patient with epilepsy.

Introduction

Levetiracetam((S)-α-ethyl-2-oxo-pyrrolidine acetamide, UCB L059) belongs to a new generation of antiepileptic drugs (AEDs), which sold as trade name Keppra to treat epilepsy. Levetiracetam is applied as monotherapy and add on therapy also (1). Levetiracetam has linear kinetic, less than 10 percent bound to plasma protein, steady-state status in blood is achieved in 48 h. Levetiracetam has no significant interaction with other AEDs. In the body 34 percent of Keppra is metabolised by enzymatic hydrolysis and 66 percent is excreted unchanged in urine with an elimination half-life of 6-8 hours (2–3). A microdialysis study, based on using freely behaving rat model, suggests that levetiracetam is transported rapidly through the blood-brain barrier and half-life of levetiracetam in cerebrospinal fluid is much higher than in plasma (4). Longer presence in cerebrospinal fluid enables patients to take levetiracetam only twice a day. Levetiracetam has an asymmetric carbon atom (Figure 1), and has two enantiomers, which have different pharmacokinetic properties. Isoherrane and co-workers have shown that no chiral inversion takes place in dogs (5).

A few studies deal with analysis of levetiracetam using chromatographic and electrophoresis methods (6–13). Only one ultra-high-performance liquid chromatographic tandem mass spectrometry (UHPLC–MS–MS) method is known in the literature (14).

In the UHPLC systems, the combination of sub-2 μm particles and high pressure resulted in shorter analysis time (15) with improvements in resolution, sensitivity and peak capacity (16). UHPLC systems can also be applied at higher linear velocity than that is used commonly in LC methods without losing efficiency significantly (17, 18).

Validation of the analytical method is important task for analysis. The analytical parameters of the guidelines of the International Conference on Harmonization (ICH), U.S. Food and Drug Administration (FDA) and U.S. Pharmacopeia (USP) were compared by Shabir et al. (19).

The aims of the current study are (i) to convert the developed HPLC method to UHPLC for analysis of levetiracetam, (ii) to develop and to optimize a simple and efficient sample preparation method, (iii) to validate the newly developed UHPLC method, (iv) to apply UHPLC method to determine levetiracetam in plasma samples of patient with epilepsy.

Experimental

Materials and reagents

Levetiracetam was kindly provided by UCB Pharma (Brain-l'Alleud, Belgium). Potassium dihydrogen phosphate (GR for analysis), sodium hydroxid (for analysis), dichloromethane (for liquid chromatography), HPLC gradient grade methanol and HPLC gradient grade acetonitrile were purchased from Merck (Darmstadt, Germany). Ultra pure water was always prepared fresh using MilliQ Synergy UV apparatus (Millipore, Billerica, MA, USA). Extrelut sorbent for liquid-liquid extraction was obtained from Merck (Darmstadt, Germany), Oasis HLB and Oasis MAX solid phase extraction columns were purchased from Waters (Budapest, Hungary).

An external standard stock solution of levetiracetam (1000 μg/mL) was prepared by dissolving 10 mg of levetiracetam in 10 mL of HPLC gradient grade methanol and stored at 4°C.

UHPLC

Analysis was performed with Waters Acquity UPLC system equipped with binary pump and photo diode array detector controlled by Empower Pro software.

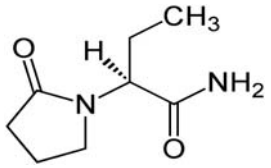

Figure 1. Chemical structure of levetiracetam.

Mobile phase was acetonitrile-phosphate buffer (pH = 6.6; 0.01 M) filtered through 0.22 μm membrane filter (La-Pha-Pack, Budapest, Hungary) (10/90 v/v). The stationary phase was Acquity BEH C18 column with a particle size of 1.7 μm (100-2.1 mm i.d.). The column temperature was maintained at 30°C. The flow rate of 0.2 mL/min was applied and the injected volume was 20 μL. The UV detection was at 215 nm.

HPLC

The HPLC system used was Merck Hitachi HPLC instrument, equipped with ternary pump (Merck Hithachi L-7100), autosampler (Merck Hitachi L-7250), UV detector (Merck Hitachi L-7400) and data station (Merck Hitachi D-7000). The system was controlled by D-7000 HSM software.

The other HPLC instrument used was equipped with binary pump (Waters 1525), autosampler (Waters 717 Plus), UV–VIS detector (Waters 2487 UV/VIS Dual Absorbance). This system was controlled by Waters Breeze Chromatography Software Version 3.30.

The mobile phase was acetonitrile–phosphate buffer (pH = 6.6; 0.01M) filtered through 0.22 μm membrane filter (La-Pha-Pack, Budapest, Hungary) (10/90 v/v). Agilent Zorbax Extend C18 column with particle size of 5 μm (250-4.6 mm i.d.) was applied as the stationary phase. The flow rate was 1 mL/min and 20 μL of the sample was injected. The column temperature was maintained at 30°C. The UV detection was performed at 210 nm.

Blood sampling

Blood sampling was carried out in National Institute of Neuroscience with Sarstedt Monovette serum tubes with clot activator. Venous blood (5 mL) samples were collected from patients' routine monitoring of AED's in the morning, before or after their first dose of Keppra. Sample tubes were centrifuged at 3500 rpm for 10 min at 25°C (MLW T52.1). Blood serum was transferred into Eppendorf tubes for further study. Blood serum samples were stored not longer as one month at −20°C and two weeks at 4°C before measurement.

Sample preparation

In the recovery study, standard stock solution of levetiracetam was added at five different concentrations to drug-free plasma samples (kindly provided by National Institute of Neuroscience, Budapest, Hungary). Three different sample preparation methods are described below.

Solid-phase extraction

Waters Oasis HLB and Oasis MAX cartridges were used and were activated in three steps. First, cartridges were washed with 2 mL of dichloromethane, followed by conditioning with 2 mL of methanol and finally equilibrated with 2 mL of water. Spiked plasma sample (500 μL) was loaded onto the cartridge and washed with 2 mL of water. The washed sample was eluted with 1 mL of methanol.

Liquid-liquid extraction on Extrelut

Extrelut cartridges were prepared in the laboratory by first conditioning with 1 mL mixture of dichloromethane and methanol (30:70, v/v, %). The prepared plasma sample (500 μL) was added onto the conditioned cartridge. After waiting period of ten minutes, the cartridge was washed with 6 mL dichloromethane. The eluent was dried at 60°C in water bath and redissolved in 700 μL mobile phase. Sample was filtered through 0.22 μm membrane filter and injected into the chromatographic system.

Protein precipitation with organic solvents

Ratio of spiked plasma sample and organic solvent (methanol or acetonitrile) was 1 to 2. Solutions were mixed and centrifuged (MLW T52. 1; 10 min, 5000 rpm) after 10 min standing. Supernatants were diluted to the half with the mobile phase and filtered through 0.22 μm membrane filter.

UHPLC (Waters UPLC) method validation

The UHPLC method was validated in terms of precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ) and linearity according to the ICH guidelines (20, 21). The robustness of the applied UHPLC method was also studied.

Precision was determined using nine independent test solutions in three different concentrations.

Accuracy of the UHPLC method was evaluated with the recovery of standards in five different concentrations from drug free plasma with three replications. The extraction was carried out as described in earlier in this work and analyzed using the UHPLC method.

LOD and LOQ for levetiracetam were determined by injecting of diluted spiked drug free plasma sample at known concentration. LOD is expressed as a concentration at 3:1 and 10:1 signal-to-noise ratio, respectively.

Linearity test was evaluated by drawing 5 point calibration curve. Drug free plasma samples were spiked with external standard stock solution in five different concentrations.

To determine the robustness of the method, the column temperature was varied by ± 1°C, the percentage of organic modifier was adjusted by ± 1% and the pH of the buffer was varied by ± 0.5 pH units.

Results and Discussion

Precision

Precision was assessed at three concentrations (2, 40, 80 μg/mL) and three replications were done at three separate occasions. The relative standard deviations were 1.98%, 1.15% and 1.47% for 2, 40, and 80 μg/mL concentrations, respectively.

Table I
Recovery Results with Different Sample Preparation Method

	Liquid–liquid extraction		Protein precipitation with methanol		SPE with Oasis HLB	
c (μg/mL)	Average recovery (%)	RSD	Average recovery (%)	RSD	Average recovery (%)	RSD
2	8.22	123.18	97.05	4.39	n.d.	-
25	22.82	58.24	98.21	3.37	13.36	4.30
50	34.51	38.04	99.70	1.2	14.25	7.52
75	41.85	38.07	100.37	1.32	21.54	8.06
100	50.05	31.11	99.46	1.43	3.52	3.77

Accuracy

Three different sample preparation methods were tested as described in the Sample preparation section. The recoveries were determined with spiked plasma samples in triplicate over the range of 2–100 μg/mL.

Oasis MAX had weak retention for levetiracetam. The recovery results with Oasis HLB cartridges were between 3 and 22 % with RSD $<$ 10 %.

The recovery of levetiracetam in liquid–liquid extraction procedure strongly depended on the concentration and the relative standard deviations were between 38 and 123 %.

Interferences could be experienced in the protein precipitation with acetonitrile sample preparatory method. When methanol was applied as an organic solvent, no interference was experienced. The recovery results were between 97.1 and 100.4%, RSD values were below 5%. Results are summarized in Table I.

A summary of advantages and disadvantages of different sample preparation methods is provided in Table II. Solid-phase extraction preparatory method was the most complicated and time consuming. In further measurements, protein precipitation with methanol was applied as the sample preparation method. This method was the simplest and used lowest amount of organic solvent.

LOD, LOQ

The limit of detection was 10 ng/mL and limit of quantification was 33 ng/mL.

Linearity

Drug free plasma was spiked with levetiracetam in five different concentrations between 2 and 100 μg/mL, which is the 80-120 % of the target interval. Extraction was carried out as described in the 'Protein precipitation with organic solvents' section. Regression equation was $y = 9765.514x$, with a multiple R^2 of 0.9997. (The p value of the Lack of fit test was 0.17)

Robustness

Full factorial experimental design (2^3) was applied to study robustness of the method. Effect of acetonitrile content was significant (with 0.004 p value). No interaction between the factors was obtained. Pareto chart, shown in Figure 2. depicts the effect of temperature, pH, acetonitrile, and interaction between these parameters on the retention time.

Table II
Advantages and Disadvantages of Different Sample Preparation Methods

Sample preparation method	Advantage	Disadvantage
Solid phase extraction (SPE)		Interference, bad recovery with high standard deviation, complicated, time consuming, most expensive
Liquid–liquid extraction (LLE)	No interference	Recovery depend on concentration, complicated
Protein precipitation with methanol	No interference, good recovery results, simplest, lowest amount of organic solvent, cheapest	

Serum samples

Five different patients' blood samples were analyzed. Protein precipitation with methanol was applied for sample preparation. Samples were measured by two different analysts with three different liquid chromatographic systems (two HPLCs and UPLC). Intermediate precision was good (RSD was below 5 %, except in case of patient 5). Results are summarized in Table III. and Figure 3. depicts a typical chromatogram of patient plasma sample obtained by UPLC and HPLC.

Comparison of HPLC and UHPLC methods

In our study a HPLC method was transferred to UPLC. Agilent Zorbax Extend C18 column with particle size of 5 μm (250-4.6 mm i.d.) belongs to Silica B category with high surface coverage.

In the UHPLC, assumption was made that BEH C18 column with particle size of 1.7 μm (100-2.1 mm i.d.) has the same or higher non-polarity, because of high coverage and hybrid technology.

In method transfer first the flow rate was adjusted. Flow rate was calculated based on the generally accepted equation (16). Equation 1. considers the difference in diameter of columns used in HPLC and UHPLC.

$$\left(\frac{d_2}{d_1}\right)^2 * F_1 = F_2 \qquad (1)$$

where: d_1 is the diameter of HPLC column; d_2 is diameter of UHPLC column; F_1 is the flow rate in HPLC system; F_2 is flow rate in UHPLC system. Based on the performed calculations, a flow rate of 0.2 mL/min was applied as flow rate in UPLC system. Some chromatographic parameters for the two liquid chromatographic systems are summarized in Table IV.

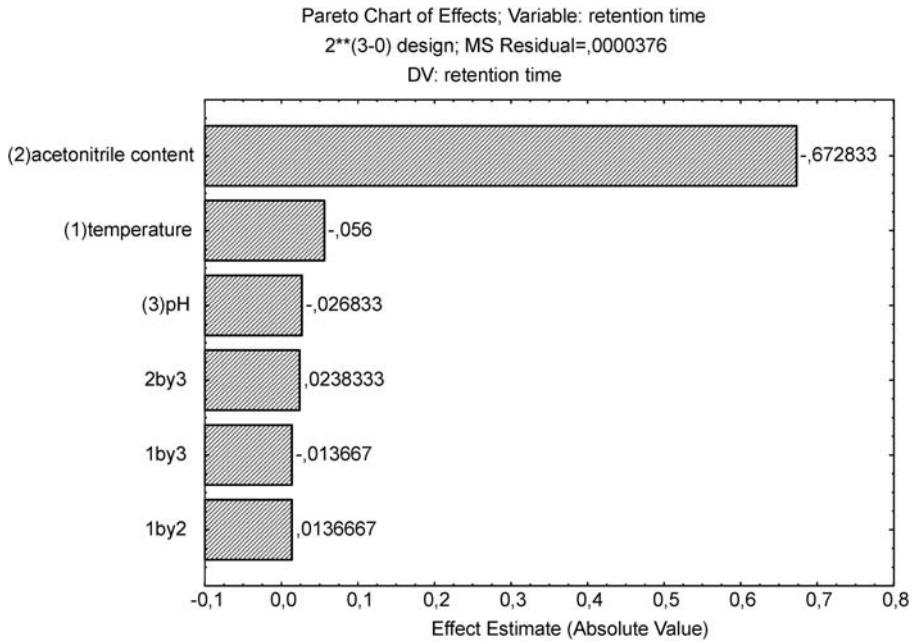

Figure 2. The Pareto chart of robustness test displays the standardized effect of the acetonitrile content of the mobile phase, the pH of the buffer and column temperature on retention time.

Table III
Results of Patients' Plasma Samples with UPLC and Two HPLC Systems.

	HPLC (μg/mL)	UPLC (μg/mL)	Other analist with other HPLC system (μg/mL)	born	mg/day	Hours between last administration and blood sampling
1. patient	24.18	24.35	22.8	1948.06.23.	1500	6
2. patient	<LOD	<LOD	<LOD	2007.09.10	1000	trough conc.
3. patient	23.73	23.56	21.2	1975.01.11	2500	5.45
4. patient	20.56	20.11	19.5	1978.08.23	1000	3
5. patient	19.38	19.75	15.9	1978.02.18	1500	3

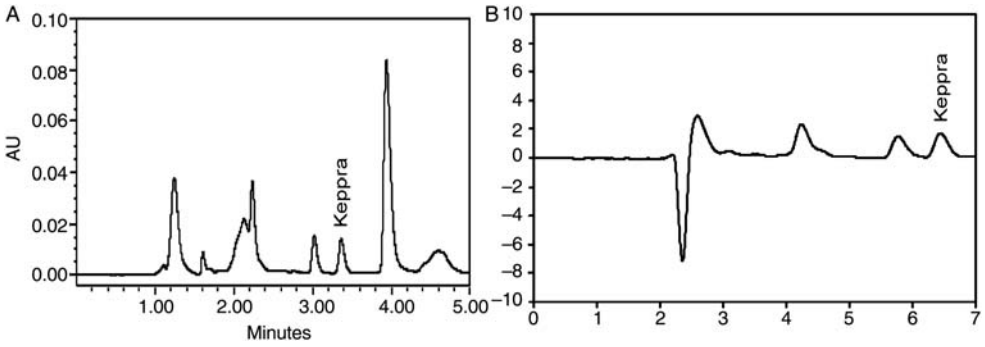

Figure 3. Typical chromatograms of patient plasma sample using protein precipitation with methanol as sample preparation method, mobile phase was acetonitrile-phosphate buffer (pH = 6.6; 0.01 M) (10/90 v/v), temperature of columns maintained at 30°C (A) BEH C18 (100−2.1mm, 1.7 μm) column with flow rate of 0.2 mL/min, UV detection was performed at 215 nm, (B) Agient Zorbax Extend C18 (250-4.6 mm, 5 μm) column with a flow rate of 1 mL/min, UV detection was performed at 210 nm.

Table IV
Some Chromatographic Parameters for UPLC and Merck Hitachi HPLC

Chromatographic parameter	UPLC	HPLC
Retention factor	2.2	1.5
Asymmetry factor	1.36	1.52
Theoretical plate number (1/m)	24900	12600
Limit of detection (ng/mL)	10	287
Limit of quatitation (ng/mL)	33	959

The retention factor was higher in the UPLC method. Additionally, the UPLC method gave high plate numbers, which were two times higher than those of observed in the HPLC method. This is in agreement with theory.

The retention time in the UPLC was also half compared to HPLC method. Both detection and quantification limits were ~30 times lower in the UPLC than the HPLC method.

Table V
Summary of Limit of Detection, Limit of Quantitation and Linearity Range of Different Methods

	UPLC–PDA	LC–UV (6)	LC–UV (7)	LC–UV (8)	LC–ESI–MS–MS (10)	LC–ESI–MS–MS (11)	LC chiral column (12)	UPLC–MS–MS (14)
LOD (μg/mL)	0.01	1.0	0.1	0.15	-	0.1	0.9	0.06
LOQ (μg/mL)	0.033	2.0	1.0	0.5	1.0	1.0	2.25	0.15
Linearity range (μg/mL)	2–100	4–80	1–75	5–100	1–40	1–50	2.25–90	0.5–150

Conclusions

The already known HPLC method for determination of levetiracetam from plasma samples was successfully converted to UHPLC. In both cases the non-polar characters of stationary phases were the same. In HPLC practice a highly covered stationary phase (Zorbax Extend C18) was used. In UHPLC method a hybrid phase (BEH C18) was applied, which has high non-polarity. The two non-polar stationary phases had similar interactions with active ingredient and matrix compounds of plasma. We demonstrated that if the column surface chemistry in HPLC and UHPLC methods is similar then the developed HPLC method could be converted to UHPLC method.

Of the three tested sample preparation procedures (liquid-liquid extraction with Extrelut, solid phase extraction and protein precipitation with organic solvents), protein precipitation with methanol was the most accurate and the simplest with less time consumption.

The transferred method from HPLC to UHPLC was validated in the assay range of 2-100 μg/mL ($R^2 = 0.9997$) with 10 ng/mL LOD and 33 ng/mL LOQ. Recovery of protein precipitation was in the range of 97.1–100.4 %. The effective plasma concentration of levetiracetam is between 0.0035 and 0.24 mM. Compared to the effective concentration range of levetiracetam and assay range of validated method, this method is sufficient to control the serum level of drug and helps the therapy. The developed UHPLC method has unique advantages e.g. the analysis time and LOD, LOQ were reduced.

A use of less sample dilution and greater injection volume (20 μL) in comparison with 2 μL in the UPLC–MS–MS, the developed UPLC–PDA method had lower LOD and LOQ than 0.06 μg/mL LOQ and 0.15 μg/mL LOD obtained in the UPLC-MS/MS method. The analytical system performance of our method and others published in the literature are summarized in Table V.

According to the pharmacokinetic parameters, the highest concentration in blood is usually reached in 1-2 hours after the last administration and $t_{1/2}$ could be measured in 6-8 hours. The levels of drug sink to LOD after 10-12 hours. TDM can be performed after 6-8 hours. In the studied patients, four serum samples were in this level, and one person had below the limit of detection of levetiracetam. Overall, the UHPLC-PDA method can be applied for therapeutic drug monitoring (TDM).

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