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Short communication

Simple and validated HPLC–UV analysis of levetiracetam in deproteinized plasma of patients with epilepsy

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ABSTRACT

We present a simple, fast and validated method for the determination of the new generation antiepileptic drug (AED) levetiracetam (LEV) in plasma of patients with epilepsy using high performance liquid chromatography (HPLC) with UV detection. Plasma sample (500 μL) pretreatment was based on simple deproteinization by methanol spiked with the internal standard (I.S.). HPLC analysis was carried out on a Synergi 4-μm Hydro-RP, 150 mm × 4 mm I.D. column. The mobile phase was a mixture of potassium dihydrogen phosphate buffer (50 mM, pH 4.5) and acetonitrile (94:6, v/v) at an isocratic flow rate of 1.5 mL/min. The UV detector was set at 205 nm. Calibration curves were linear (mean correlation coefficient = 0.999) over a range of 4–80 μg/mL. The quantitation limit was 2 μg/mL and the absolute recovery was >90% for LEV and the I.S. Both intra and interassay precision and accuracy were lower than 7.5%. The chromatographic run lasted 13 min. The present procedure omitting expensive solid phase or time-consuming liquid–liquid extraction and drying steps is cheaper, faster and simpler than mostly published analytical methods for levetiracetam. Applied to a large population of patients with epilepsy this assay proved very practical in our therapeutic drug monitoring setting (TDM).

1. Introduction

Levetiracetam (LEV), [(*S*)-α-ethyl-2-oxo-pyrrolidine acetamide] (Keppra®, UCB Pharma, Braine-l'Alleud, Belgium) is a new antiepileptic drug (AED) chemically related to the nootropic agent piracetam [1] approved for the treatment of partial onset seizures with or without secondary generalisation and as adjunctive therapy for myoclonic seizures. Although the role of therapeutic drug monitoring (TDM) for LEV has not been fully established, measuring plasma drug concentration can be useful in assessing compliance and managing patients in situations associated with pharmacokinetic alterations (i.e. pregnancy), in pathological states such as renal impairment and in specific age groups (children and the elderly) [2,3]. Different HPLC methods for the determination of LEV in human plasma have been reported, coupled with UV [4–7] or diode array detection [8,9], mostly after sample pretreatment by expensive solid-phase extraction [4] or time-consuming liquid–liquid extraction procedures [5,7,9]. The availability of simple, accurate and inexpensive analytical assays is crucial for the successful use of TDM in clinical practice. Levetiracetam spiked plasma sample preparation by different kinds of deproteinization before HPLC-diode array detection was first explored by Pucci et al. [8] and subsequently applied to patient samples analysis by HPLC–UV [6].

Here we describe a new rapid and simple validated HPLC–UV method for measurement of LEV in plasma of patients with epilepsy which further simplifies both the chromatographic apparatus [6,8] and plasma pretreatment [6] of previously reported methods and is convenient for application in a routine AED TDM setting.

2. Experimental

2.1. Reagents and standards

Levetiracetam and the internal standard (I.S.) UCB 17025 (α-2,2-trimethyl-5-oxo-1 pyrrolidine acetamide) (Fig. 1), were kindly provided by UCB Pharma (Brain-l'Alleud, Belgium). Gradient grade acetonitrile and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a MilliQ Gradient A10 apparatus (Millipore, Billerica, MA, USA).

Stock solutions (1 mg/mL) of LEV and I.S. and subsequent dilutions (100 μg/mL, working solution) of LEV were prepared by dissolving LEV in ultrapure water and UCB 17025 in methanol. All solutions were prepared monthly and stored at 4 °C.

Plasma standards for the calibration curve of 4.0, 10.0, 20.0, 40.0 and 80.0 μg/mL for LEV were prepared by pipetting suitable

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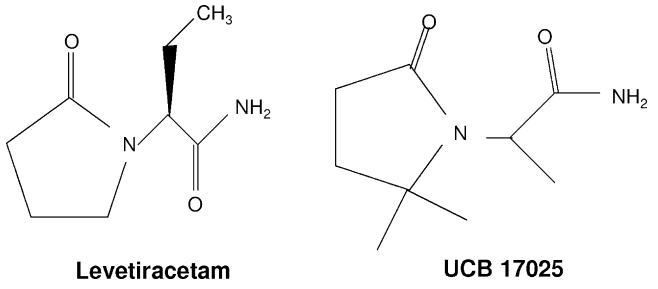

Fig. 1. The chemical structures of levetiracetam and UCB 17025.

amounts of working LEV solution (for the 4.0 μg/mL calibrator) and of stock drug solution (for all the remaining calibrators) to 500-μL aliquots of blank pooled plasma and then treated exactly as patients' specimens.

2.2. Chromatographic apparatus and conditions

The HPLC system consisted of a Series 200 liquid chromatograph, a Series 200 UV/vis spectrophotometric detector, set at 205 nm, and a Series 200 autosampler connected by a model 600 link chromatography interface to the TotalChrom chromatography workstation. All the equipment was purchased from Perkin Elmer, Norwalk, CA, USA.

Chromatographic separations were performed with a Synergi 4-μm Hydro-RP, 150 mm × 4.6 mm I.D. column (Phenomenex, Torrance, CA, USA) protected by a C_{18} Securityguard precolumn (Phenomenex) and a graphite filter (ESA, Chelmsford, MA, USA). The mobile phase was a mixture of potassium dihydrogen phosphate buffer (50 mM, pH 4.5) filtered through a 0.22-μm membrane filter (GS type, Millipore) and acetonitrile (94:6, v/v). The mobile phase was sparged with helium and the isocratic flow rate was set at l.5 mL/min.

2.3. Blood sampling and plasma processing

Venous blood samples (5 mL) were drawn from patients for routine monitoring of AED at 8 AM, before their first morning dose of AEDs, transferred into heparinized tubes (8 IU heparin/mL blood) and centrifuged at 1500 × *g* for 10 min at 4 °C. Plasma was separated, transferred into test tubes and stored at 4 °C for a maximum of 2 weeks. Five-hundred-microliter plasma aliquots were deproteinized by addition of 1.5 mL methanol spiked with I.S. (0.067 mg/mL), vortexed for 1 min and then centrifuged at 1500 × *g* at 4 °C for 20 min. Ten microliters of the clean upper layer were injected into the chromatographic system.

2.4. Method specificity

Standard solutions of several commonly coprescribed AEDs, their metabolites and benzodiazepines were injected to check for possible interferences (Table 1). Blank plasma from six pools was tested for endogenous interferences. Furthermore, a series of plasma samples from patients with epilepsy not taking LEV and treated with commonly prescribed AED and non-AED cotherapies (including antidepressants, hypnotics, antipsychotics, different types of antibiotics) were analyzed to check for drugs which could potentially interfere with LEV determination.

Table 1
List of drugs checked for levetiracetam assay interference

Antiepileptic drugs
Carbamazepine
Carbamazepine-epoxide
Ethosuximide
Felbamate
Gabapentin
Lamotrigine
Oxcarbazepine and monohydroxycarbamazepine
Phenobarbital
Phenytoin
Pregabalin
Primidone
Tiagabine
Topiramate
Valproic acid
Vigabatrin
Zonisamide
Benzodiazepines
Clobazam
Clonazepam
Diazepam
Lorazepam
Nitrazepam
Norclobazam

2.5. Method validation

Calibration curves for LEV were run on each analysis day ($n = 11$) over 4 months. The analyte-to-I.S. peak area ratios were plotted against LEV matched concentration added to the blank plasma. The calibration curves were calculated by the least square method. Linearity was assessed by determining the coefficient of correlation (r) of the points of the curves.

For assay precision and accuracy assessment, blank plasma pools were spiked with an appropriate volume of working or stock LEV solutions to yield concentrations of 4.0, 20.0, and 80.0 μg/mL, corresponding to the lower, middle and upper points of the calibration curve, separated into 500-μL aliquots and stored frozen at −20 °C. The precision of the method was assessed by determining the relative standard deviation (R.S.D. = 100 × S.D./mean) at the three plasma LEV concentrations within the same analysis ($n = 6$, intraday precision) and in triplicate over a series of six analyses ($n = 18$, interday precision).

The accuracy of the method was determined by comparing the means of the calculated concentrations at the three plasma concentrations for LEV with the nominal concentrations (percentage differences), within the same analysis ($n = 6$, intraday accuracy) and in triplicate over a series of six analyses ($n = 18$, interday accuracy).

The absolute recovery of both LEV and I.S. was calculated as the ratio of the drug peak area from deproteinized blank plasma spiked with LEV, at the three above-mentioned concentrations, or with the I.S. (25 μg/mL)-to-peak area obtained from the injection of LEV and I.S. standard solutions, at the same theoretical concentrations reconstituted in methanol over a series of six analyses.

The lower limit of quantitation (LOQ) was defined as the lowest quantifiable concentration with an associated R.S.D. < 20% [10]. The precision and accuracy at the LOQ were determined both intraday ($n = 6$) and interday (triplicate samples over six analyses, $n = 18$).

The lower limit of detection (LOD) was calculated as three times the baseline noise [10].

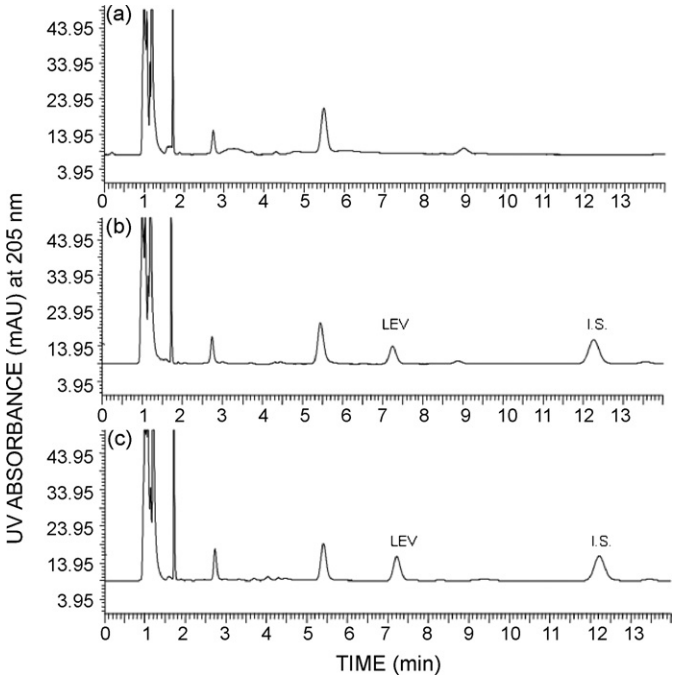

Fig. 2. Chromatograms obtained by injecting 10 μL of (a) deproteinized blank plasma; (b) blank plasma spiked with LEV, 20.0 μg/mL and I.S., 25.0 μg/mL; and (c) plasma specimen of a patient treated with levetiracetam (1000 mg/die) and clobazam (5 mg/die): LEV, 26.1 μg/mL. LEV, levetiracetam; I.S., internal standard.

3. Results and discussion

3.1. Chromatography

During the development of the assay, different mobile phases were evaluated. The mixture already described (potassium dihydrogen phosphate buffer, 50 mM, pH 4.5 and acetonitrile, 94:6, v/v) provided optimal separation of LEV and I.S. with mean ± S.D. (n = 11) retention times of 7.21 ± 0.03 min for LEV and 12.2 ± 0.05 min for the I.S. Typical chromatograms obtained from a blank plasma, a blank plasma spiked with a standard mixture of LEV and I.S., and a typical patient plasma are reported in Fig. 2. There were no endogenous interferences in the assayed analytes' elution region for any of the blank pools tested. None of the possibly coprescribed drugs tested was detected over a 22-min run (Table 1). From analyses of plasma of patients with epilepsy not taking LEV and treated with commonly prescribed AED and non-AED cotherapies no interfering peak was detected.

3.2. Validation

Calibration curves showed a linear and reproducible correlation between LEV plasma concentrations and matched analyte-to-I.S. peak area ratios. Mean equation (±S.D., n = 11) of the regression line was: $y = -0.00563\ (\pm 0.0018) + 0.0238\ (\pm 0.00125)x$, $r = 0.9990\ (\pm 0.0004)$, where x is LEV concentration, expressed in μg/mL, y is the analyte-to-I.S. peak area ratio expressed in arbitrary area units and r is the correlation coefficient.

The results of precision and accuracy analyses for LEV are reported in Table 2. The R.S.D.s for both intra and interassay precision were below 7.5% for the whole concentration range. Deviation of the means of the measured concentrations from their nominal concentrations (intra and interassay accuracy) was within 6.5%. The LOQ was set at 2.0 μg/mL (Table 2). The LOD was 1.0 μg/mL. Mean absolute recovery for LEV was 90.0 ± 0.08% at a plasma drug concentration of 4.0 μg/mL, 91.1 ± 0.08% at 20 μg/mL and 100.0 ± 0.06% at 80 μg/mL; recovery for the I.S. (25 μg/mL) was 97.5 ± 0.05% (n = 6).

3.3. TDM application

The assay was applied to the quantification of LEV in 174 plasma specimens of patients with epilepsy referred to our laboratory over 4 months, treated with LEV (dosage range, 250–4000 mg/die) combined with different AED cotherapy and the results were compared with those obtained by an extraction procedure with dichloromethane [5] routinely applied in our laboratory. Steady-state LEV plasma trough concentrations ranged from 2.5 to 88.8 μg/mL. Results obtained by deproteinization vs reference liquid/liquid extraction were highly correlated: the linear regression equation for correlation was $y = 0.989x + 0.361$, $r = 0.992$, with a standard error of 2.07, where y is the LEV plasma concentration by deproteinization and x is the LEV concentration by liquid/liquid extraction.

Compared with most HPLC methods for LEV plasma analysis published so far [4,5,7,9] this assay significantly simplifies sample purification by omitting time-consuming and expensive solid-phase or liquid/liquid extraction and drying steps, with reduced risks of analytical errors.

One of the main advantage of the present LEV analysis is only one step of sample clean-up before HPLC–UV injection, which further simplifies a previously proposed HPLC–UV procedure for LEV determination in human plasma based on deproteinization by perchloric acid and extraction of unpolar components by cyclohexane before injection into a porous graphitic carbon column [6]. The work of Pucci et al. [8] systematically evaluated the feasibility of different clean-up procedures for the HPLC-diode array analysis of LEV in human plasma, including deproteinization by addition of organic solvents or by formation of insoluble salts. Pretreatment with both acetonitrile and perchloric acid was discarded due to the very low extraction efficiency, while purification by methanol allowed an absolute recovery around 97%, which is in line with our results. The injection of a low aliquot of deproteinized specimens and the adoption of the filter combined with the guard column provides highly effective protection of our analytical system: a change of filter after about 120 injections and the preguard column after 250

Table 2
Precision and accuracy of levetiracetam assay

Amount added to blank plasma (μg/mL)	Intraday (n = 6)			Interday (n = 18)		
	Calculated concentration, mean ± S.D. (μg/mL)	Precision, R.S.D. (%)	Accuracy (%)	Calculated concentration, mean ± S.D. (μg/mL)	Precision, R.S.D. (%)	Accuracy (%)
2.0 (LOQ)	2.13 ± 0.09	2.2	6.5	2.07 ± 0.15	7.2	3.5
4.0	3.99 ± 0.21	5.2	−0.2	4.03 ± 0.20	4.9	0.7
20.0	20.29 ± 0.67	3.3	1.4	20.20 ± 0.76	3.8	1.0
80.0	78.18 ± 1.71	4.6	−2.3	81.05 ± 5.00	6.2	1.3

Precision (R.S.D.%) = 100 × S.D./mean; accuracy (%) = 100 × [(mean concentration found − known concentration)/known concentration]; interday (n = 18) = triplicate samples, over a series of six analyses on different days; LOQ = limit of quantitation.

avoids increased column back pressure and maintains a good chromatographic efficiency (about 1200 deproteinized samples injected to date).

Another strong point of our method is the high selectivity of the HPLC–UV detection allowing LEV determination in plasma of patients also receiving complex AED comedication in about 13 min with no potentially interfering peak carryover. The method quantitation range is adequate for LEV purposes even in patients receiving low daily dosages, as the LOQ of the assay is well above the lowest concentration value of currently proposed tentative "reference range" for LEV of 12–46 μg/mL [3]. The statistical validation shows a good intra and interassay precision and accuracy within the whole concentration range and an optimal extraction efficiency. Finally, results of LEV TDM in patients with epilepsy obtained by this assay are highly correlated with those obtained after a validated extraction with dichloromethane [5].

4. Conclusion

The proposed method proved to possess adequate specificity, sensitivity, accuracy and precision for a reliable determination of LEV in patients with epilepsy. The minimal sample pretreatment allows a large series of patient samples to be processed in a short time, a very advantageous task in a TDM setting. Moreover, the simple reversed-phase HPLC–UV chromatographic apparatus means the method can be adopted even in laboratories lacking sophisticated analytical equipment.

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