

## Short communication

## Determination of levetiracetam in human plasma with minimal sample pretreatment

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Received 3 December 2004; accepted 31 January 2005

Available online 16 February 2005

### Abstract

We here present a method for the routine quantification of the novel antiepileptic drug levetiracetam in human serum by HPLC–UV. The procedure is very easy, quick, inexpensive and rugged. The sample preparation consists only in the precipitation of serum proteins by perchloric acid and extraction of unpolar components by cyclohexane. The aqueous phase containing the analyte levetiracetam is injected onto a porous graphitic carbon analytical HPLC-column and separated by gradient elution with diluted phosphoric acid/acetonitrile. Detection is carried out at a wavelength of 205 nm. The calibration function is linear in the range of 1–75 µg/ml. The detection limit is 0.1 µg/ml. Using four quality control sample concentrations, the inter-day relative standard deviations (R.S.D.) are lower than 3% and the accuracies are better than 6%. The respective inter-day values are: R.S.D. < 4% and accuracies better than 2%. Frequently co-administered antiepileptic drugs do not interfere with the assay. The method has been successfully applied to patient samples.

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**Keywords:** Levetiracetam; Antiepileptic drugs; Porous graphitic carbon; HPLC

### 1. Introduction

Levetiracetam (Fig. 1) is a novel antiepileptic drug which is structurally and mechanistically dissimilar to other antiepileptic drugs [1]. Its pharmacokinetic profile is linear with respect to dosage, its bioavailability is close to 100%, it undergoes only insignificant hepatic metabolism to inactive metabolites, it does not induce hepatic enzymes and about 91% of the dose is excreted via the renal route [2]. Therefore, it is close to a drug with ideal pharmacokinetic properties. Nevertheless, it is recommended to monitor the plasma concentrations of levetiracetam to optimize the therapeutic effect, especially in patients with renal impairment, in the elderly where the half-life of the drug is extended [3] and in children, where the half-life is shortened [4].

There are only a few papers published reporting therapeutic drug monitoring methods of levetiracetam. Three of them

employed HPLC with UV-detection [5–7], and two methods were using GC with NPD-detection [6,8]. Microemulsion electrokinetic chromatography with UV-detection was utilized in one method [9], but it lacks suitable sensitivity. Two methods facilitating chiral separation of the *S*- and *R*-enantiomer of levetiracetam, one utilizing GC–MS and the other HPLC–UV, were published recently [10,11]. These methods were designed to investigate in dogs the pharmacokinetic and pharmacodynamic properties of the two enantiomers separately. For routine therapeutic drug monitoring in men, these methods were not appropriate. In all but one of the methods [7], sample preparation with SPE or liquid–liquid extraction is necessary. Pucci et al. [7] evaluated the feasibility of protein precipitation as the only sample preparation step in comparison to SPE. They concluded, that protein precipitation is a suitable and fast sample preparation for measuring routine patient samples.

All published HPLC-methods used reversed phase stationary phases. Because of the very polar nature of levetiracetam, a reasonable retention was only achieved with very low fractions of organic modifiers in the mobile phase. We here

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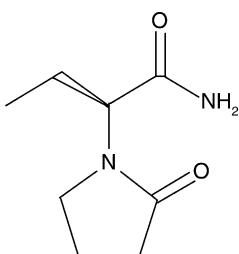


Fig. 1. Chemical structure of levetiracetam.

present a new method for the determination of levetiracetam in human serum which utilizes only protein precipitation as sample preparation and separates the analyte from endogenous substances and other antiepileptic drugs on a porous graphitic carbon analytical column. This type of column leads to better retention, very sharp and symmetrical peak shapes and exhibits a very good selectivity for levetiracetam.

## 2. Experimental

### 2.1. Instrumentation

The HPLC-instrumentation consists of an Agilent 1100 system (Waldbronn, Germany) comprising a degasser, a quaternary pump, a thermostatted column compartment and a variable wavelength detector. Data are collected and analyzed by an Agilent ChemStation software package, version 9.3. The chromatographic separation of the analyte takes place on a Thermo Hypercarb 150 mm × 4.6 mm (5 µm particle size) analytical column protected with a 10 mm × 4 mm guard column, filled with the same material (Thermo Electron Cooperation, Runcorn, UK).

### 2.2. Chemicals and solutions

Levetiracetam was obtained as “Keppra” film tablets containing 250 mg levetiracetam each (UCB, Kerpen, Germany). Acetonitrile and cyclohexane were purchased in HPLC-grade from Merck (Darmstadt, Germany). Ultra pure water was produced in our laboratory by a Barnstead EASYPure UV system (Werner, Leverkusen, Germany). External calibration and quality control samples were purchased from Chromsystems (Munich, Germany). All other chemicals were obtained in analytical grade or better.

### 2.3. Calibration samples

A levetiracetam stock solution was prepared by dissolving one “Keppra” 250 mg tablet in 100 ml water. The dissolution was accelerated by sonification of the mixture for 10 min. From this stock solution, 0.5 ml were diluted with 2 ml water to obtain a working solution with a concentration of 0.5 mg/ml. By spiking drug free human plasma with the working solution, calibration samples in the concentra-

tion range from 1 to 75 µg/ml were obtained. This calibration range covers the therapeutic concentrations of levetiracetam in patient samples.

The accuracy of the levetiracetam stock solution was verified by the comparison with external calibration samples (lyophilized human serum) obtained from Chromsystems (Munich, Germany, order no. 24003, lot no. 114), which were reconstituted according to the manufacturers instructions.

### 2.4. Quality control samples

External quality control samples (lyophilized human serum) were purchased from Chromsystems in two concentration levels with target values of 8.54 µg/ml and 51.09 µg/ml (order no. 0087/0088, lot no. 114). The control samples were reconstituted according to the manufacturers instructions and were taken through the same sample preparation process as unknown samples from patients. In order to get two additional levels, quality control samples were diluted 1:1, v/v before sample preparation with drug free human serum to yield concentrations of 4.27 µg/ml and 25.55 µg/ml.

### 2.5. Sample preparation

From a patient sample, calibration sample or quality control sample, 200 µl were mixed with 100 µl 0.33 M HClO<sub>4</sub> for protein precipitation and 200 µl cyclohexane to extract unpolar interferences. The samples were vortexed for 10 s and the precipitated proteins were separated by centrifugation at 9000 × g for 5 min. From the clear aqueous phase, about 100 µl were transferred into autosampler vials with microliter inserts and forwarded to the HPLC-system.

### 2.6. Chromatographic conditions

After injection of 5 µl of the prepared sample, the separation of levetiracetam was accomplished by gradient elution. Solvent A consisted of 0.423% H<sub>3</sub>PO<sub>4</sub> (5 ml H<sub>3</sub>PO<sub>4</sub> 85% in 11 water), solvent B of acetonitrile. The gradient ratio of solvent B started at 5%, raised in 8 min to 30% and was subsequently put to 100% for 4 min to elute strongly retained substances. After each run, a re-equilibration time of 4 min was necessary. The flow rate was 1 ml/min and the column temperature was 35 °C. The detection wavelength was set to 205 nm. The retention time of levetiracetam was 6.8 min under the described conditions.

## 3. Results and discussion

### 3.1. Sample preparation

The only sample preparation step necessary in this method is protein precipitation. This is accomplished by the addition of perchloric acid. This has several advantages over solid phase or liquid-liquid extraction [5–8], since this procedure

is very easy, quick and cheap compared with an extraction procedure. The protein precipitation with organic solvents like methanol or acetonitrile [7] has also a drawback, because a mixture rich in organic solvent is produced, which in the case of levetiracetam has a higher solvent strength than the mobile phase of the HPLC-separation. Injection of such a mixture leads to distortion of the chromatographic progress and therefore to peak broadening. The extract produced from protein precipitation by perchloric acid has a very low solvent strength and a pH-value in the range of the mobile phase. Therefore, the peaks are refocused after injection, leading to very narrow and symmetric peak shapes in the chromatogram. The extraction recovery is found to be  $98.6 \pm 0.56\%$  ( $n=6$ , levetiracetam peak areas from spiked human plasma extracts compared with water containing levetiracetam concentrations according to 100% theoretical extraction yield). Such a nearly quantitative recovery and the usage of a modern automatic HPLC-injector makes the addition of an internal standard to the samples redundant. The low extraction yield reported by Pucci et al. [7] in the perchloric acid protein precipitation procedure cannot be confirmed in our work.

The addition of cyclohexane to the samples in our sample preparation procedure fulfills the purpose of extracting unpolar plasma constituents. Levetiracetam is not extracted by cyclohexane in acidic medium and therefore the quantification is uninfluenced. However, unpolar substances

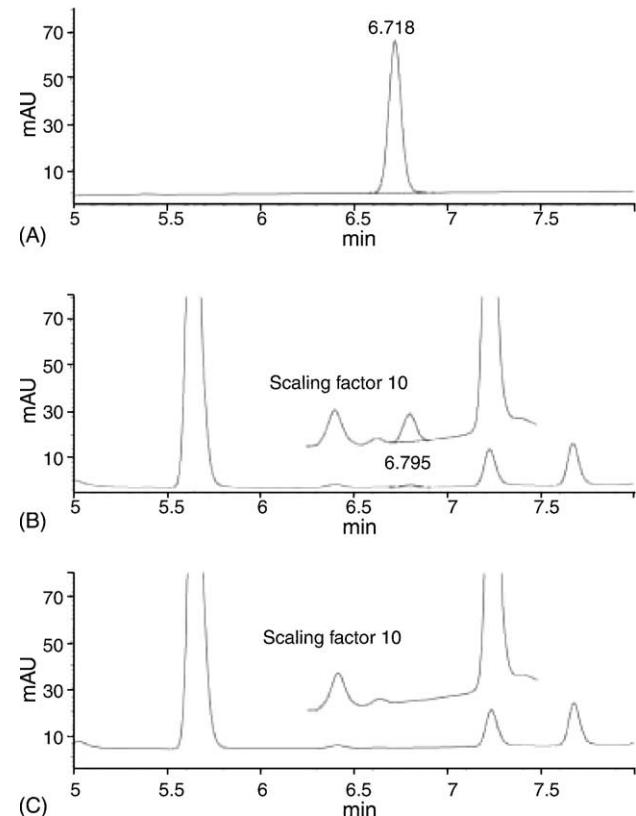


Fig. 2. Typical chromatograms from (A) water containing  $50 \mu\text{g}/\text{ml}$  levetiracetam, (B) the lowest calibration level ( $1 \mu\text{g}/\text{ml}$ ) from drug free human plasma and (C) drug free human plasma without levetiracetam.

are very difficult to elute from a porous graphitic carbon column, and the repetitive loading of the column with such substances would result in a quick decline of the column performance (product info Thermo Hypercarb columns). With the extraction of such substances before injection, we have analyzed several hundred samples without any maintenance of the chromatographic system, and we have observed no performance loss so far.

### 3.2. Chromatography

The chromatographic separation of levetiracetam on a porous graphitic carbon column leads to a sharp and symmetrical peak (Fig. 2A). Since levetiracetam lacks chromophores (Fig. 1), it can only be detected with sufficient sensitivity at very short wavelengths, in our case 205 nm. At this spectral range, UV-detection is very unspecific and selectivity has to be achieved by chromatographic separation. As it can be seen, in spiked serum samples (Fig. 2B and C) and in patient samples with antiepileptic co-medication (Fig. 3A–C), despite the non sophisticated sample preparation, no interferences from endogenous substances or the co-administered drugs

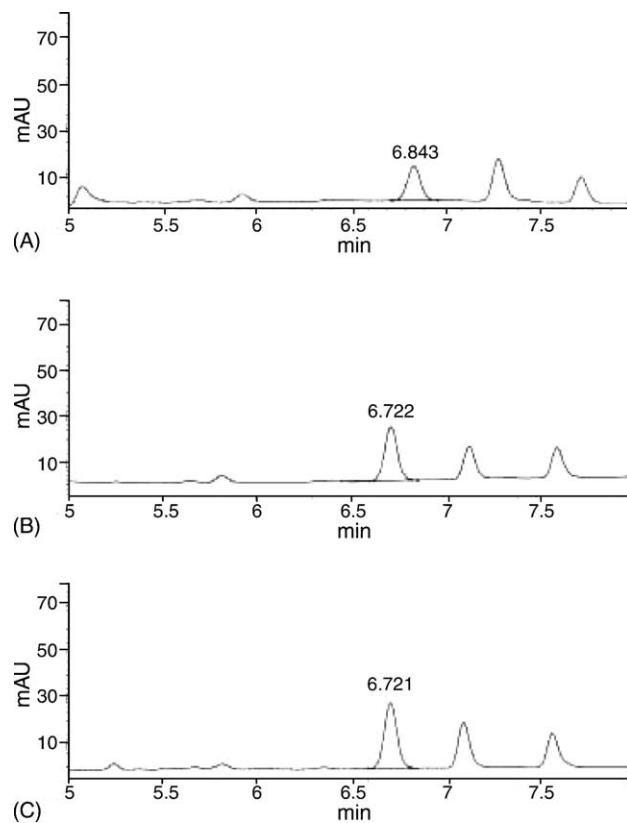


Fig. 3. Chromatograms of patients receiving co-medication of other antiepileptic drugs beside levetiracetam: (A) dosage: levetiracetam 375 mg/day, lamotrigine 100 mg/day, phenobarbital 150 mg/day, levetiracetam concentration:  $12.0 \mu\text{g}/\text{ml}$ ; (B) dosage: levetiracetam 1500 mg/day, primidone 500 mg/day, levetiracetam concentration:  $17.2 \mu\text{g}/\text{ml}$ ; (C) dosage: levetiracetam 1500 mg/day, lamotrigine 100 mg/day, levetiracetam concentration:  $20.7 \mu\text{g}/\text{ml}$ .

Table 1  
Intra- and inter-day precision and accuracy

Sample	<i>n</i>	Expected concentration ( $\mu\text{g}/\text{ml}$ )	Measured concentration ( $\mu\text{g}/\text{ml}$ )	R.S.D. (%)	Accuracy (%)
<b>Intra-day</b>					
Level 1	10	4.27	4.17	0.33	-3.17
Level 2	10	8.54	8.07	2.56	-5.54
Level 3	10	25.55	24.46	0.24	-4.25
Level 4	10	51.09	49.19	0.34	-3.71
<b>Inter-day</b>					
Level 1	8	4.27	4.32	3.11	1.06
Level 2	8	8.54	8.50	3.12	-0.51
Level 3	8	25.54	25.38	2.02	-0.63
Level 4	8	51.09	49.65	1.34	-0.88

are observable. This selectivity is achieved by the unique retention mechanism provided by the material of the stationary phase. A similar selectivity cannot be obtained by the usage of standard RP-phases, where, to our experience, patient samples occasionally are not quantifiable due to endogenous interferences. Other antiepileptic drugs, such as carbamazepine, oxcarbazepine, phenytoin, phenobarbital, lamotrigine, primidone, ethosuximide, valproic acid, sulfamethoxazole and their respective main metabolites were tested for co-elution. Under the described conditions, none of the drugs or their metabolites are interfering with levetiracetam.

### 3.3. Validation

The calibration is carried out using a standard solution of levetiracetam obtained from a "Keppra" 250 mg tablet. This procedure is obviously not optimal but unavoidable because to our knowledge no pure levetiracetam is available commercially. To verify the accuracy of the so obtained levetiracetam calibration samples, they were compared to external calibration samples purchased by Chromsystems. The difference in the calibration slopes determined by these two calibration sample sets was 5.4% and can therefore be regarded as minor. Furthermore, in everyday analysis of patient samples, external quality control samples were included, which yielded very accurate results (Table 1). The reason not to use the calibration samples marketed by Chromsystems in everyday analysis is the limited calibration range of maximal 26.6  $\mu\text{g}/\text{ml}$ , which is not sufficient to quantify all types of patient samples.

The calibration function is linear in the range of 1–75  $\mu\text{g}/\text{ml}$  ( $n=7$ ). The weighed least square parameters are: intercept =  $0.775 \pm 0.204$  and slope =  $6.080 \pm 0.0053$  with a correlation coefficient better than 0.9999. The limit of quantification is defined as the lower limit of the calibration range, i.e. 1  $\mu\text{g}/\text{ml}$ . This limit has not been pushed further because the quantification of levetiracetam at concentrations lower than about 1  $\mu\text{g}/\text{ml}$  lacks therapeutic relevance. The limit of detection has been found to be 0.1  $\mu\text{g}/\text{ml}$  at a signal-to-noise ratio of 3.

The intra- and inter-day precision and accuracy values were determined in four concentration levels at 4.27  $\mu\text{g}/\text{ml}$ ,

8.54  $\mu\text{g}/\text{ml}$ , 25.54  $\mu\text{g}/\text{ml}$  and 51.09  $\mu\text{g}/\text{ml}$ . The results are summarized in Table 1. As can be seen, all relative standard deviations and accuracy deviations are below 10%, which satisfies the requirements for bioanalytical method validation [12].

## 4. Conclusion

We here present a method for the determination of levetiracetam in patient samples. The method is easy, cheap (no usage of expensive and laborious SPE), quick (only about 10 min hands-on time per sample) and rugged. The usage of a porous graphitic carbon analytical HPLC-column leads to unique selectivity which results in the absence of any interferences from endogenous substances or co-administered antiepileptic drugs. The method is suitable for routine therapeutic drug monitoring and has been successfully applied to a large number of patient samples.

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