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# Quantification of levetiracetam in plasma of neonates by ultra performance liquid chromatography–tandem mass spectrometry

Maren I. Blonk[a], Bart C. van der Nagel[a], Liesbeth S. Smit[b,c], Ron A.A. Mathot[a,*]

[a] Department of Hospital Pharmacy, Erasmus University Medical Center, 's-Gravendijkwal 230, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands
[b] Department of Neonatology, Erasmus University Medical Center, Sophia Children's Hospital, Dr. Molewaterplein 60, 3000 CB, Rotterdam, The Netherlands
[c] Department of Pediatric Neurology, Erasmus University Medical Center, Sophia Children's Hospital, Dr. Molewaterplein 60, 3000 CB, Rotterdam, The Netherlands

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ABSTRACT

A sensitive and specific method using ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was developed for the determination of levetiracetam (LEV) in plasma of neonates. A plasma aliquot of 50 μl was deproteinized by addition of 500 μl methanol which contained 5 μg/ml UCB 17025 as an internal standard. After centrifugation, 50 μl of supernatant was diluted with 1000 μl of 0.1% formic acid–10 mM ammonium formate in water (pH 3.5) (mobile phase solution A) and 2 μl was injected onto the UPLC-system. Compounds were separated on a Acquity UPLC BEH $C_{18}$ 2.1 mm × 100 mm column using gradient elution with mobile phase solution A and 0.1% formic acid in methanol (mobile phase solution B) with a flow rate of 0.4 ml/min and a total runtime of 4.0 min. LEV and the internal standard were detected using positive ion electrospray ionization followed by tandem mass spectrometry (ESI-MS/MS). The assay allowed quantification of LEV plasma concentrations in the range from 0.5 μg/ml to 150 μg/ml. Inter-assay inaccuracy was within ±2.7% and inter-assay precision was less than 4.5%. Matrix effects were minor: the recovery of LEV was between 97.7% and 100%. The developed method required minimal sample preparation and less plasma sample volume compared to earlier published LC–MS/MS methods. The method was successfully applied in a clinical pharmacokinetic study in which neonates received intravenous administrations of LEV for the treatment of neonatal seizures.


## 1. Introduction

Levetiracetam (LEV), (S)-α-ethyl-2-oxo-1-pyrrolidine acetamide (Fig. 1), is a relatively new antiepileptic drug (AED). It is structurally unrelated to the traditional AEDs (e.g. phenytoine, carbamazepin and valproic acid) and has different antiepileptogenic properties. Although the exact mechanism is unknown, binding to synaptic vesicle protein 2A (SV2A) may play a role [1]. U.S. Food and Drug Administration (FDA) approved the drug as adjunctive therapy in the treatment of partial onset seizures (age ≥ 4 years), myoclonic and tonic–clonic seizures. In Europe it is furthermore approved as monotherapy for partial onset seizures [2,3]. The efficacy of LEV has, besides these approved indications, been evaluated in both children and adults with status epilepticus [4,5]. LEV exhibits linear pharmacokinetics and is not bound to serum proteins. About 66% of the administered dose is excreted renally as unchanged drug. Unlike other AEDs that are metabolized, the metabolism of LEV does not include the cytochrome P450 system. Approximately 24% of LEV undergoes enzymatic hydrolysis producing inactive metabolites [6]. Furthermore LEV exhibits a favorable safety profile and minimal drug interactions [2].

Seizures are the most common neurological problem in neonates and can be a major therapeutic challenge in neonatal intensive care units. The treatment of neonatal seizures has changed little over the last 50 years. Phenobarbital is still the standard first-line AED in neonatal seizures, but is effective in less than 50% of the cases [7–9]. With the recent development of a parenteral formulation of LEV, this compound may be used in neonates as well. Since information with respect to pharmacokinetics and safety of LEV in neonates is lacking, an open-label pilot study is currently being performed in the Sophia Children's Hospital of the Erasmus University Medical Center in the Netherlands. A pharmacokinetic study in neonates is complicated since only a limited volume of blood can be obtained. The availability of a sensitive bio-analytical method is therefore a prerequisite.

Several methods have been developed for quantification of LEV in human plasma, e.g. gas chromatography (GC) with nitrogen-phosphorus detection or mass spectrometry [10,11], high-performance liquid chromatography (HPLC) with UV-detection [12–14], and more recently liquid chromatography with

* Corresponding author. Tel.: +31 10 7033202; fax: +31 10 7032400.
*E-mail addresses:* miblonk8@xs4all.nl (M.I. Blonk), r.mathot@erasmusmc.nl (R.A.A. Mathot).

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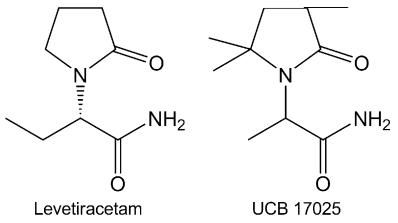


**Fig. 1.** Chemical structures of levetiracetam and UCB 17025.

tandem mass spectrometry (LC–MS/MS) [15–17]. In comparison with these methods quantification of LEV in plasma may further be enhanced by application of ultra-performance liquid chromatography (UPLC). UPLC is performed with columns packed with sub-2 μm particles which results in reduced plate heights and thus improved resolution and sensitivity compared to conventional liquid chromatography. UPLC provides a greater signal to noise ratio and shorter runtimes with minimal peak broadening [18,19]. It may therefore be more suitable for high-throughput analysis. The combination of UPLC with the selective MS/MS detector results in a powerful technique which can be used for quantitative drug analysis in small volumes of patient material with minimal sample pre-treatment [18,20].

In this study a sensitive and simple UPLC–MS/MS method was developed for the quantification of LEV in small volumes of plasma. The method was validated and applied to samples from two neonates participating in a pharmacokinetic study of LEV.

## 2. Experimental

### 2.1. Chemicals and materials

LC–MS grade water and LC–MS grade methanol were purchased from Biosolve (Valkenswaard, the Netherlands) and used as mobile phases. Other aqueous solutions were prepared with ultrapure water from the apparatus MQSynergy 185 from Millipore S.A. (Molsheim, France). Levetiracetam ($C_8H_{14}N_2O_2$) and the internal standard UCB 17025 ($C_9H_{16}N_2O_2$) were kindly provided by UCB Pharma S.A. (Brussels, Belgium). Formic acid ULC/MS grade and methanol absolute HPLC supra-gradient were purchased from Biosolve (Valkenswaard, the Netherlands). Ammonium formate puriss. p.a. for mass spectroscopy was purchased from Sigma–Aldrich (Steinheim, Germany). Levetiracetam Serum Control Levels I and II were purchased from Chromsystems (München, Germany). Drug-free EDTA-plasma was obtained from the department of Haematology of the Erasmus Medical Center (Rotterdam, the Netherlands).

### 2.2. Standard solutions and QC samples

Two separate stock solutions of levetiracetam (LEV) in ultrapure water were prepared with a concentration of 5 mg/ml. These stock solutions were alternately diluted with water to produce standard working solutions with LEV concentrations of 5, 20, 50, 100, 250, 500, 1000 and 1500 μg/ml. The standard working solutions were divided and stored at −80 °C until use. Calibration standards were prepared by spiking drug-free human EDTA-plasma with a standard working solution (1:9; v/v) producing LEV plasma concentrations of 0.5, 2, 5, 10, 25, 50, 100 and 150 μg/ml. The plasma calibration standards were used immediately after preparation.

The component UCB 17025, α-2,2-trimethyl-5-oxo-1 pyrrolidine acetamide (Fig. 1), was used as an internal standard (IS). UCB 17025 was dissolved and diluted in methanol to prepare a working solution with a final concentration of 5 μg/ml. The IS working solution, also used for protein precipitation during sample preparation, was stored at −20 °C until use.

For the preparation of internal quality control (QC) samples for intra- and inter-assay comparisons, a separate stock solution of LEV was prepared in water (5 mg/ml) and further diluted in water to a working solution (1000 μg/ml). Internal QC samples were prepared in four concentration levels by diluting this working solution with drug-free human EDTA-plasma in volumetric flasks. The obtained plasma concentrations were Lower Limit of Quantification (LLOQ) 0.5 μg/ml, low (L) 1.5 μg/ml, medium (M) 35 μg/ml and high (H) 120 μg/ml. For the preparation of external QC samples, Levetiracetam Serum Control Levels I and II from Chromsystems were dissolved in 2 ml water. The obtained concentrations were 10.1 μg/ml (Level I) and 55.1 μg/ml (Level II). The QC samples were analyzed and the remaining solution was divided and stored at −80 °C until further use.

### 2.3. Sample preparation

To 50 μl of plasma, 500 μl IS working solution (5 μg/ml UCB17025 in methanol) was added in an Eppendorf centrifuge tube. The sample was vortexed vigorously for at least 30 s to precipitate the proteins. After centrifugation at 13,200 rpm for 6 min, 50 μl of the supernatant was transferred to a glass autosampler vial to which 1000 μl of 0.1% formic acid–10 mM ammonium formate in water (pH 3.5) (mobile phase solution A) was added. The composition of the final extract was similar to the initial composition of the mobile phase at the start of the run. The samples were vortexed and 2 μl was injected into the UPLC–MS/MS system.

### 2.4. Instrumentation and chromatographic conditions

The UPLC–MS/MS system consisted of a Waters Acquity Ultra Performance LC coupled to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The analytical column was an Acquity UPLC BEH $C_{18}$ 2.1 mm × 100 mm column with 1.7 μm particle size, to which a 0.2 μm pre-column filter unit was added (Waters Ltd., Dublin, Ireland). The mobile phase was a gradient of solution A (0.1% formic acid–10 mM ammonium formate in LC–MS grade water, pH 3.5) and solution B (0.1% formic acid in LC–MS grade methanol) with an initial composition of 10% B for 0.8 min. Mobile phase composition changed linearly from 10% B to 90% B in 1.7 min. After 0.7 min the composition was switched back to 10% B and left to equilibrate for 0.8 min. Total runtime was 4 min. The flow rate was 0.4 ml/min with a column temperature of 50 °C and an injection volume of 2 μl (10 μl loop, partial loop with needle overfill). The autosampler temperature was set at 15 °C. Analytes were detected via MS/MS with an electrospray ionization (ESI)-interface in positive multiple reaction monitoring (MRM)-mode. Mass transitions of levetiracetam ($m/z$ 171.0 → 125.9) and of UCB 17025 ($m/z$ 185.0 → 139.9) were optimized. The ESI-MS/MS operating parameters used in this study are listed in Table 1. Data were acquired using Masslynx V4.1 software and processed using Quanlynx V4.1 (Waters Corp., Milford, MA, USA).

### 2.5. Validation procedures

A validation of the assay in human plasma was performed according to FDA guidelines for bio-analytical method validation, including linearity, accuracy, precision, selectivity, ion suppression and stability [21]. Matrix effects were determined according to the method from Matuszewski et al. [22]. Eight non-zero plasma calibration standards were prepared and analyzed in duplicate in

**Table 1**
Settings of the Quattro Premier XE tandem quadrupole mass spectrometer.

| Parameter | Setting | |
|---|---|---|
| Run duration (min) | 4 | |
| Capillary voltage (kV) | 1 | |
| Source temperature (°C) | 130 | |
| Desolvation temperature (°C) | 350 | |
| Desolvation gas flow (L/h) | 700 | |
| Cone gas flow (L/h) | 100 | |
| Collision gas flow (ml/min) | 0.35 | |
| | Levetiracetam | UCB 17025 (IS) |
| Q1 *m/z* | 171.0 | 185.0 |
| Q3 *m/z* | 125.9 | 139.9 |
| Dwell time (s) | 0.1 | 0.1 |
| Cone voltage (V) | 11 | 11 |
| Collision energy (eV) | 15 | 15 |

6 analytical runs. Weighted linear regression was performed on the ratio of the peak-area of the analyte and the peak-area of the internal standard versus the LEV plasma concentration. The best fit was selected after exploration of different regression models and weighting factors. The final curves were constructed by IS calibration and weighted ($1/x$) least square linear regression with the origin included. The deviations from the nominal concentration should be within ±20% for the LLOQ and within ±15% for the other concentrations.

Accuracy and precision were established by analyzing five replicates of QC samples in three analytical runs and duplicates of QC samples in three additional analytical runs. All runs were performed on different days in a total period of 4 weeks. Inaccuracy was defined as the deviation from the nominal concentration in percentage by quantifying QC samples on a freshly prepared calibration curve. In total 6 different QC samples were used: LLOQ 0.5 μg/ml, low 1.5 μg/ml, medium 35 μg/ml, high 120 μg/ml, Level I 10.1 μg/ml and Level II 55.1 μg/ml. The deviation (inaccuracy) should be within ±15% except at the LLOQ concentration, where it should be within ±20%. The coefficient of variation (CV) represents the intra- and inter-assay precision. Precisions (CV = standard deviation/mean × 100%) should be within ±15% (±20% at LLOQ). The LLOQ and Limit of Detection (LOD) were calculated according to the following equations: $\mathrm{LOD} = \bar{y} + 3 * s_b$ and $\mathrm{LLOQ} = \bar{y} + 10 * s_b$ ($\bar{y}$ =average response of blank plasma ($n = 10$), $s_b$ = standard deviation of blank plasma). The 10 blank plasma samples were measured consecutively in a single run. The calculated LOD and LLOQ should be below the proposed LLOQ used in this assay (0.5 μg/ml). Carry-over was tested by injecting a processed blank matrix sample sequentially after injecting the highest calibration standard. The response at the retention times of LEV should be less than 20% of the mean response of a LLOQ sample.

Plasma and solvent components in the ionization chamber cause batch specific ion suppression or enhancement, leading to inter- and intra-patient signal variability. To investigate whether endogenous matrix components interfered with the assay, matrix effects were evaluated in two different ways. During the development of the assay, ion suppression was investigated by continuous infusion of two separate solutions of LEV and IS (1 μg/ml and 0.25 μg/ml in methanol via post-column tee-connection using a syringe pump, flow 5 μl/min). Subsequently 10 plasma samples of different lots (including heparinised, haemolytic and lipemic plasma) were injected onto the column. Chromatograms were checked for suppression or enhancement of the constant signal caused by compounds that elute from the column [23]. Additionally, matrix effects were quantified as previously described by Matuszewski et al. [22]. In short, the peak areas of LEV and IS were determined in three different sets of samples: pure standards prepared in water and diluted in mobile phase solution A (set 1), six different batches of plasma spiked before precipitation (set 2), and six different batches of plasma spiked after precipitation (set 3). Samples were spiked with low (L), medium (M) and high (H) concentrations of LEV in duplicate and spiked with IS to obtain a concentration of 0.2 μg/ml in the final extract. Precipitation was performed by adding 500 μl methanol to 50 μl plasma. Subsequently, 50 μl of the supernatant was diluted with 1000 μl of mobile phase solution A. Recovery (RE) was defined as the percentage of the average peak area of samples spiked after precipitation compared to before precipitation. Matrix effects (ME) were similarly defined as the percentage of the signal (peak area) of post-extraction spiked plasma samples compared to spiked aqueous samples. Process efficiency (PE) was defined as the product of RE and ME divided by 100, i.e. the overall signal in area of spiked plasma compared to an aqueous standard solution. Average values (%) of RE, ME and PE were calculated over the six plasma batches together with coefficients of variations of RE and ME.

Stability of LEV in spiked human EDTA plasma samples was assessed for different conditions and time periods. The freeze–thaw stability was determined by comparing the stability samples that had been frozen (−80 °C) and thawed three times, with freshly prepared QC samples. Bench-top stability (8 h ambient temperature) and stability at 2–8 °C for 24 h and 7 days of plasma samples were investigated in triplicate and compared to freshly prepared QC samples. Stability at −80 °C for 4 weeks was investigated in a similar way in duplicate. The re-injection stability of the autosampler (15 °C) was determined in triplicate after 24 h and compared with the initial concentrations. In addition the same procedure was followed to determine the stability of the reconstituted samples (final extract) stored for 7 days at 2–8 °C. The above described stability experiments were performed at two concentration levels (1.5 and 120 μg/ml). All stability evaluations were based on mean back-calculated concentrations of freshly prepared and analyzed QC samples. The analyte is considered stable in biological matrix or extracts if 85–115% of the reference concentration is recovered.

### 2.6. Clinical application

The developed assay was applied to samples from neonates participating in a pharmacokinetic study of LEV at the Sophia Children's Hospital in the Netherlands. The Medical Ethics Committee of the Erasmus University Medical Center approved the study protocol. Written informed consent was obtained from both parents. Inclusion criteria were electrographical epileptic seizures (multiple seizures (>1 in 30 min) lasting at least 8 s or status epilepticus) diagnosed by electroencephalography (EEG), gestational age of >37 weeks, birth weight >1500 g, seizures refractory to phenobarbital up to 40 mg/kg or refractory to phenobarbital and subsequently midazolam up to 0.5 mg/kg, and the presence of an arterial catheter. LEV therapy (20 mg/kg intravenous infusion over 5 min) was started under continuous EEG monitoring. In case of continuing seizures an additional dose of LEV 20 mg/kg was given approximately 10 min after the end of the first infusion. Blood (300 μl) was sampled from an arterial catheter before the infusion and at 5, 15, 20, 30, 60 min, 2, 4, 8, 12, 18, 24, 36, 48, 60 and 78 h after the start of the infusion. After centrifugation, plasma was stored at −80 °C until analysis. Plasma levels of LEV were measured in 50 μl plasma according to the assay described. Individual pharmacokinetics parameters were assessed by fitting multi-compartment pharmacokinetic models to measured plasma concentrations using WinNonlin® Version 5.2 (Pharsight Corp., Mountain View, CA, USA). The best model was selected on basis of plots of weighted residuals versus time and the Akaike information criterion (AIC) [24].

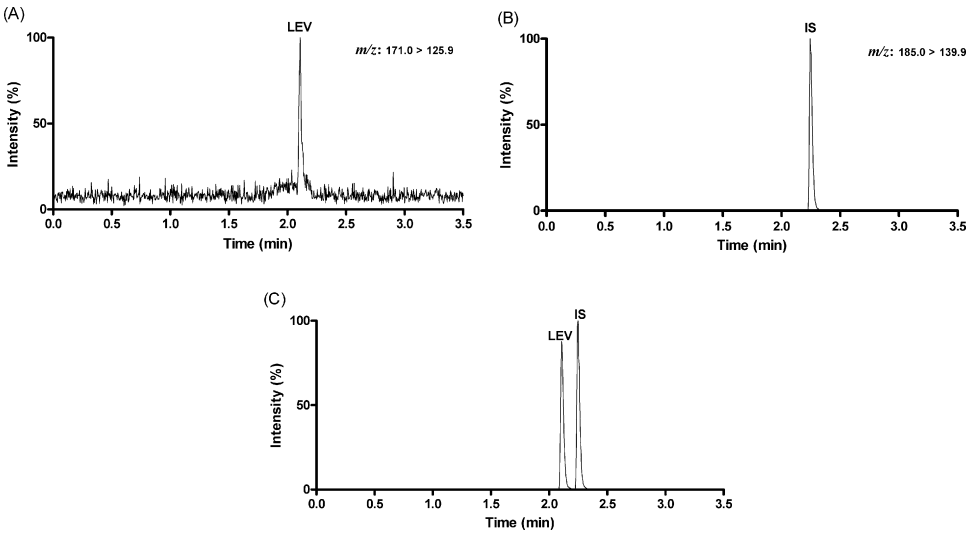


**Fig. 2.** Representative UPLC–MS/MS chromatograms of spiked human plasma with levetiracetam (LEV) at LLOQ (A, 0.5 μg/ml, $t_r$ 2.10 min), internal standard (IS) UCB 17025 (B, 50 μg/ml, $t_r$ 2.24 min) and a patient plasma sample 2 h after levetiracetam iv 40 mg/kg (C, 49 μg/ml).

## 3. Results and discussion

### 3.1. Mass spectrometry and chromatographic conditions

In order to optimize ESI conditions for LEV and IS, full scan ESI mass spectra were required in the positive detection mode and tuning parameters were varied. Mass transitions of LEV (*m/z* 171.0 → 125.9) and of UCB 17025 (*m/z* 185.0 → 139.9) were selected based on their stability and high intensity. We encountered problems with early fragmentation of LEV to *m/z* 154 due to the settings of the cone voltage. By lowering the cone voltage from 20 to 11 V, LEV remained intact in the first quadrupole (Q1). The settings of the mass spectrometer used in this method are listed in Table 1.

Chromatographic conditions were optimized to achieve acceptable peak shape (asymmetry factor LEV 2.1 and IS 1.8) and runtime. The most critical chromatographic parameter was the composition of the mobile phase. Gradient elution was chosen with 0.1% formic acid–10 mM ammonium formate in water (pH 3.5) as solution A and 0.1% formic acid–methanol as solution B. We experienced less background noise and improved resolution with a buffered eluent, by adding 10 mM ammonium formate to 0.1% formic acid in water (pH 3.5). The initial composition of the mobile phase consisted of 10% B which was gradually increased to 90% B and then switched back to elute the remaining compounds from the column. The retention time of LEV and IS were 2.10 and 2.24 min respectively. The column was kept at 50 °C because this resulted in better peak shape due to the decrease of the viscosity of the mobile phase at higher temperatures. In addition back pressure of the column was reduced resulting in an operating pressure well under the maximum operating pressure even if the column is older or contaminated. Further elevation of column temperature was not evaluated since this shortens column lifetime. The flow rate was increased to a value (0.4 ml/min) which still allowed optimal ionisation. Volume of injection (2 μl) was as low as possible to prevent in-source fragmentation.

Preferably an IS is chosen that exhibits similar chemical and physical properties as the studied analyte. This should minimize any differences in recovery due to sample pre-treatment or differences in chromatographic behaviour. UCB 17025 is structurally very similar to LEV, as both are pyrrolidinone amides (Fig. 1). It has been used as IS in other analytical assays to quantify LEV [25]. UCB 17025 is not used in clinical practice and therefore will not be co-administered to patients and subsequently cause problems when quantifying LEV.

### 3.2. Sample preparation

Protein precipitation with methanol and a mixture of methanol–acetonitrile (10:90; v/v) was compared. The influence of the added volume of organic solvent was investigated as well. Protein precipitation by adding 500 μl methanol to 50 μl plasma gave the best results in terms of ion suppression and recovery. 50 μl supernatant was diluted with 1000 μl of aqueous mobile phase (solution A) to reduce the percentage of organic solvent in the final extract and thereby diminishing the elutropic effect of methanol. Due to the 21-fold dilution, the composition of the final extract was almost similar to the composition of mobile phase at the start of the run. The dilution was furthermore necessary to prevent saturation of the ion source at the ULOQ (150 μg/ml). Plasma matrix effects were effectively eliminated by the 231-fold dilution of the patient sample. This dilution does not reduce the clinical applicability of the method, since therapeutic plasma concentrations of LEV are relatively high (range from 12 to 46 μg/ml) [26] and the developed method is very sensitive.

In previous reports both protein precipitation and SPE have been described [14–17]. When compared to protein precipitation SPE produces cleaner extracts and requires minimal or no sample dilution. SPE is however laborious and time-consuming and often requires a larger volume of plasma. The latter is a disadvantage when only a limited volume is available as in neonatal pharmacokinetic studies. Pucci et al. compared SPE and protein precipitation and concluded that SPE procedures are best suited for the analysis of LEV in pharmacokinetic studies as application of SPE provided the highest sensitivity [14]. However, the combination of UPLC and MS/MS in the present study provided such a high sensitivity that is was possible to use a large ratio of the volumes of methanol and plasma for precipitation as well as a large dilution factor before injection. Despite the 231-fold dilution the LLOQ (0.5 μg/ml) of the developed method was the same or even lower than in previously reported methods [15–17].

### 3.3. Validation

Selectivity is the ability to differentiate the analyte from endogenous and exogenous components. There were no discernable

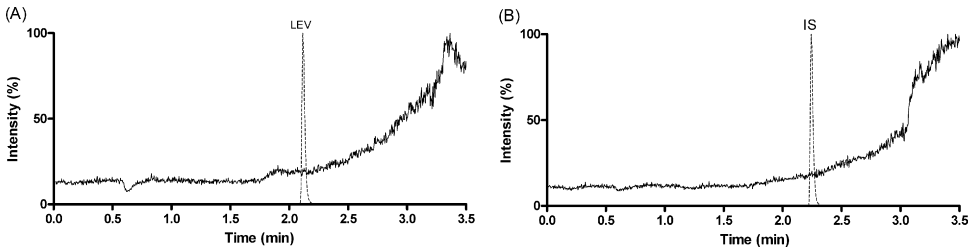


**Fig. 3.** Representative chromatogram to study ion suppression in blank plasma sample for levetiracetam (A, infusion LEV 1 μg/ml) and the internal standard (B, infusion IS 0.25 μg/ml).

interfering components in plasma judging from a comparison between blank plasma spiked with LEV at LLOQ (A), blank plasma spiked with IS (B) and a patient sample (C), see Fig. 2. Additionally when optimizing the method, ion suppression was studied in 10 different lots of plasma. Besides blank human EDTA-plasma we used plasma samples from patients using AEDs like valproic acid and carbamazepin, as well as heparinised plasma, haemolytic plasma, lipemic plasma and two different lots of plasma from neonates. None of the tested lots of plasma showed ion suppression or enhancement by interfering endogenous components due to matrix effects or by exogenous components, at the retention times of LEV and IS. A representative chromatogram to study ion suppression of LEV and IS in blank plasma is shown in Fig. 3. From this, we concluded that the combination of protein precipitation, UPLC and MS/MS detection via multiple reaction monitoring provides sufficient specificity and selectivity.

The calibration curves provided a reliable linear response in the LEV plasma concentration range from 0.5 to 150 μg/ml. The correlation coefficients ($r^2$) of the 1/$x$-weighted calibration curves were in the range of 0.9987–0.9996 ($n$ = 6, mean 0.9993). The linear regression equation of a representative analytical run was: $y$ = 0.018723*$x$ + 5.111*10E−5 ($y$ = response ratio peak area LEV to IS, $x$ = concentration LEV in μg/ml). Plots of weighted residuals versus concentrations did not indicate non-linearity. Initially we observed problems obtaining a linear response because of a lower total ion current than expected at high concentrations. The diminished response was probably due to unwanted in-source fragmentation occurring at high concentrations of LEV. This problem was solved when an extra diluting step was added and the injection volume was lowered to 2 μl. Despite the low sample volume (50 μl) and the dilution steps, sensitivity was still sufficient with an estimated LOD and LLOQ of respectively 0.06 and 0.15 μg/ml, which are well below the LLOQ used in this assay that is set at 0.5 μg/ml. Jain et al., Guo et al. and Matar et al. reported LC-MS/MS methods for LEV with LLOQs ranging from 0.5 to 1.0 μg/ml when determined in plasma volumes of 100 and 200 μl [15–17]. In the present study sensitivity is higher with a LLOQ of 0.5 μg/ml obtained with a smaller plasma volume (50 μl).

At all concentration levels of a total of six different calibration curves processed in duplicate, the deviation of measured concentration from the nominal concentration was within acceptable limits with values between −6.2% and 8.9% and an average of ±2.7%. A carry-over test was performed during the validation procedures. No interfering peaks were detected in a processed blank plasma sample injected after the highest calibration standard and the response was well below 20% of the response of LLOQ.

The results for the intra- and inter-assay precision and inaccuracy are summarized in Table 2. The intra-assay inaccuracy (% deviation), presented for one run ($n$ = 5), was 4.2% for the LLOQ and within ±5.1% for the other concentrations. The intra-assay precision (%CV) was ≤2.3% for all concentrations. The tests for intra-assay inaccuracy and precision were repeated in two additional runs (both $n$ = 5). Results were similar to those presented in Table 2 with intra-assay inaccuracy within ±5.6% and precision ≤5.3% for LLOQ and ≤3.1% for the other concentrations. Inter-assay inaccuracy (% deviation) and precision (%CV) were assessed in 6 different runs over the course of four weeks ($n$ = 21). Inaccuracy was within ±2.7% and precision was ≤4.5% for all concentrations. Intra- and inter-assay precision and inaccuracy were well ≤15% for a broad concentration range of LEV, including LLOQ.

**Table 2**
Assay performance data for levetiracetam.

| Nominal conc. (μg/ml) | Mean calculated conc. (μg/ml) | Inaccuracy (% deviation) | Precision (%CV) |
|---|---|---|---|
| *Intra-assay*[a] | | | |
| 0.500 | 0.521 | 4.2 | 2.3 |
| 1.50 | 1.50 | 0.3 | 1.9 |
| 10.1* | 9.77 | −3.3 | 1.8 |
| 35.0 | 33.8 | −4.2 | 1.7 |
| 55.1* | 53.0 | −3.8 | 1.1 |
| 120 | 114 | −5.1 | 2.0 |
| *Inter-assay*[b] | | | |
| 0.500 | 0.507 | 1.5 | 4.5 |
| 1.50 | 1.50 | 0.1 | 4.3 |
| 10.1* | 10.1 | −0.3 | 3.5 |
| 35.0 | 34.2 | −2.4 | 3.7 |
| 55.1* | 54.0 | −1.9 | 3.4 |
| 120 | 117 | −2.7 | 3.5 |

*Abbreviations*—Conc.: concentration; CV: coefficient of variation.
[a] $n$ = 5 (one run).
[b] $n$ = 21 (six different runs performed in a 4-week period).
* External QC levels.

Matrix effects were evaluated and quantified for LEV and IS as previously described (Table 3) [23,24]. Six different lots of blank plasma were tested. Minor matrix effects were observed for both LEV and IS; recovery for LEV was between 97.7% and 100%. The %CV calculated for ME and RE was under 15% for both analytes.

The stability of LEV in plasma and final extract has been described before [16,17]. We evaluated the stability of LEV in plasma at bench-top (8 h), at 2–8 °C (24 h and 7 days), at −80 °C (1 month) and after three freeze–thaw cycles. Furthermore we evaluated the stability in the final extract in the autosampler (15 °C) for 24 h and at 2–8 °C for 7 days. The results are presented in Table 4. LEV is stable in plasma at ambient temperature up to 8 h, at 2–8 °C up to 7 days, at −80 °C up to 1 month and can safely undergo

**Table 3**
Matrix effects, recovery and process efficiency for the assay of LEV and IS in plasma ($n$ = 6).

| | Levetiracetam (nominal concentrations) | | | IS |
|---|---|---|---|---|
| | 1.50 μg/ml (L) | 35.0 μg/ml (M) | 120 μg/ml (H) | 0.2 μg/ml |
| ME % (%CV) | 108.0 (3.4) | 114.0 (1.5) | 110.8 (2.1) | 100.4 (2.8) |
| RE % (%CV) | 100.0 (3.1) | 99.4 (1.7) | 97.7 (2.7) | 112.9 (3.4) |
| PE % | 107.9 | 113.4 | 108.3 | 113.3 |

*Abbreviations*—ME: matrix effects; RE: recovery; PE: process efficiency; CV: coefficient of variation.

**Table 4**
Stability data for levetiracetam.

| Matrix | Conditions | Ref. conc. (μg/ml) | Found conc. (μg/ml) | Deviation (%) | CV (%) |
|---|---|---|---|---|---|
| Plasma | Ambient, 8 h | 1.50 | 1.48 | −1.9 | 5.5 |
| | | 114 | 113 | −0.9 | 3.0 |
| Plasma | 2–8 °C, 24 h | 1.50 | 1.41 | −6.5 | 4.3 |
| | | 114 | 112 | −1.6 | 1.5 |
| Plasma | 2–8 °C, 7 days | 1.50 | 1.52 | −0.7 | 4.7 |
| | | 114 | 114 | 0.3 | 3.3 |
| Plasma | −80 °C, 1 month | 150 | 1.43 | −5.6 | 6.9 |
| | | 114 | 117 | 3.0 | 0.1 |
| Plasma | 3 freeze (−80 °C)/thaw cycles | 1.50 | 1.46 | 0.7 | 3.9 |
| | | 114 | 114 | 0.1 | 5.3 |
| Final extract | Autosampler, 15 °C, 24 h | 1.50 | 1.52 | 1.1 | 2.9 |
| | | 114 | 119 | 4.2 | 0.9 |
| Final extract | 2–8 °C, 7 days | 1.50 | 1.45 | −3.4 | 3.2 |
| | | 114 | 112 | −1.3 | 3.0 |

*Abbreviations*—Ref.: Reference; conc.: concentration; CV: coefficient of variation.

three freeze/thaw cycles. Long-term stability studies in plasma at nominally −80 °C are ongoing. Furthermore, autosampler stability was established, indicating that the final extracts can be left in the autosampler (set at 15 °C) and re-injected after at least 24 h. The final extracts can also be safely stored at 2–8 °C for 7 days. A greater concern is probably the stability of LEV in whole blood. LEV in whole blood has been said to undergo in vitro hydrolysis, which will result in lower concentrations being measured (decline of 11% in 2 days) [27]. Therefore in our study we separated plasma from whole blood immediately after the arrival of the samples at the laboratory and stored the samples at −80 °C.

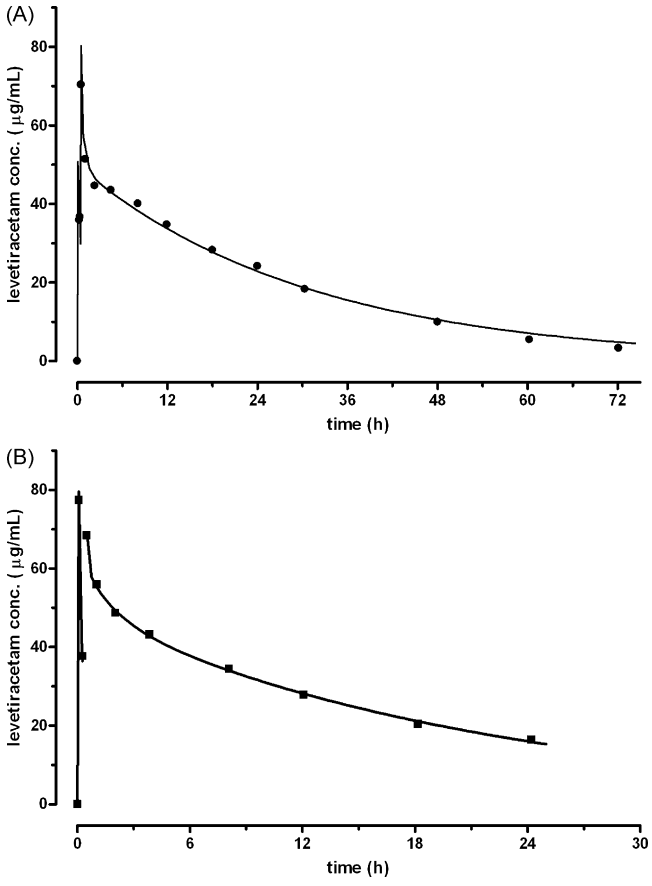


**Fig. 4.** Concentration–time profiles of levetiracetam (LEV) in two neonates receiving intravenous infusions of 20 mg/kg LEV during 5 min (A, 64 mg; B, 74 mg). Due to continuing seizures both patients received an additional dose of 20 mg/kg LEV 10 min after the end of the first infusion. The symbols represent the measured plasmaconcentrations and the solid line the fitted 3-compartment pharmacokinetic model.

## 4. Clinical application

The developed assay was applied to plasma samples of two neonates participating in a pharmacokinetic study with LEV. The neonates received intravenous doses of respectively 64 and 74 mg LEV during 5 min due to seizures caused by birth asphyxia. Because of continuing seizures a second dose was administered 10 min after the end of the infusion of the first dose. On basis of the plots of weighted residuals versus time and the Akaike information criterion, a 3-compartment pharmacokinetic model was selected to describe the time profile of LEV plasma concentration (Fig. 4) [24]. The following individual pharmacokinetic parameters were estimated: central volume of distribution 0.71 and 0.71 L, steady-state volume of distribution 2.5 and 2.7 L, clearance 0.083 and 0.14 L/h, first distribution half-life 0.017 and 0.052 h, second distribution half-life 0.36 and 0.54 h, elimination half-life 21 and 14 h for the neonate receiving two administrations of 64 (Fig. 4A) and 74 mg (B) LEV respectively.

## 5. Conclusion

The development, validation and application of an UPLC–MS/MS method for the quantitative analysis of LEV in plasma are described. LEV was extracted from human EDTA-plasma by a simple and fast protein precipitation method with methanol. A linear calibration curve was obtained in the LEV plasma concentration range from 0.5 to 150 μg/ml LEV. There were no apparent matrix effects and recovery was high. The assay requires only 50 μl plasma volume and is easy to perform with minimal sample preparation. The method is selective, sensitive, accurate, precise, reproducible, and was applied successfully in clinical pharmacokinetic research.

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