High-performance liquid chromatography with time-programmed fluorescence detection for the quantification of Levofoxcin in human plasma and cerebrospinal fluid in adults with tuberculous meningitis

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ABSTRACT

An accurate and reliable high-performance liquid chromatography with time-programmed fluorescence detection was developed and validated to measure levofoxcin in human plasma and cerebrospinal fluid (CSF). After solid phase extraction process using Evolute® ABN 96 fixed well plate; levofoxcin and internal standard-enoxacin were separated using a mobile phase consisting of phosphate buffer 10 mM with 0.025% triethylamine pH 3.0 - acetonitrile (88:12, v/v) on a Purosphere RP-8e column (5 μm, 125 × 4.0mm) at a flow rate of 1.2 mL/min at 35 °C. The excitation/emission wavelengths were set to 269/400 nm and 294/500 nm, for enoxacin and levofoxcin, respectively. The method was linear over the concentration range of 0.02 to 20.0 μg/mL with a limit of detection of 0.01 μg/mL. The relative standard deviation of intra-assay and inter-assay precision for levofoxcin at four quality controls concentrations (0.02, 0.06, 3.0 and 15.0 μg/mL) were less than 7% and the accuracies ranged from 96.75% to 101.9% in plasma, and from 93.00% to 98.67% in CSF. The validated method was successfully applied to quantify levofoxcin in a considerable quantity of plasma (826) and CSF (477) samples collected from 232 tuberculous meningitis patients, and the preliminary intensive pharmacokinetics analysis from 14 tuberculous meningitis patients in Vietnam is described in this paper.

1. Introduction

Tuberculous meningitis (TBM) is the most dangerous form of tuberculosis, causing severe morbidity and mortality in both children and adults. Even with the introduction of antibiotic treatment for tuberculosis, the death rate for TBM remains high at around 67% and 25% respectively for human immunodeficiency infected (HIV +) and uninfected (HIV-) Vietnamese adult patients [1,2]. Outcome from TBM might be improved by antibiotics with high dose of rifampicin (RIF; 15 mg/kg) and an added fift drug, levofoxcin (LEV; 20 mg/kg). This recently completed RCT shown that the intensified regimen was not associated with an improvement in treatment outcomes [4]. To understand the reasons for the lack of clinical effect, we wanted to analyze the relationship between clinical outcomes and drug concentrations in blood and CSF. Knowledge of the drug exposure concentration at the infectious site (brain) and the ratio of AUCLEV in CSF to AUCLEV in plasma should help to elucidate the trial (RCT) at two sites in Ho Chi Minh City, Viet Nam, to assess the efficacy of an intensive anti-tuberculosis treatment regimen for TBM [3]. The regimen consisted of standard doses of isoniazid (INH), ethambutol or streptomycin and pyrazinamide (PZA) in combination with high dose of rifampicin (RIF; 15 mg/kg) and an added fifth drug, levofoxcin (LEV; 20 mg/kg). This completely recent RCT showed that the intensified regimen was not associated with an improvement in treatment outcomes [4]. To understand the reasons for the lack of clinical effect, we wanted to analyze the relationship between clinical outcomes and drug concentrations in blood and CSF. Knowledge of the drug exposure concentration at the infectious site (brain) and the ratio of AUCLEV in CSF to AUCLEV in plasma should help to elucidate the
relationship between pharmacokinetics and pharmacodynamics of LEV in TB patients, and explain the clinical results.

LEV is a synthetic broad-spectrum antibacterial agent for oral and intravenous administration and is a concentration-dependent antibiotic whose effective bacteria-killing activity depends on the ratio of maximum concentration \((C_{\text{max}})\) to minimum inhibitory concentration (MIC) [5]. Measuring LEV concentration in plasma and CSF is not only helpful in making effective drug dosage regimens but also in assessing the CSF penetration of LEV in patients with TBM.

Many studies have described high-performance liquid chromatography (HPLC) methods with ultraviolet (UV) [6–9] or fluorescence (FL) detection [10–17] for the determination of LEV or other fluoroquinolones (FQ) simultaneously in human plasma [6–10,12,13,16–18]; serum [11,14,15,19]; CSF [9,18]; broncho-alveolar lavage [8]; dialysate [12,17]; soft tissue [11] and urine [20]. Recently, Sung Joong Lee, Sung Chul Shin et al. developed a liquid chromatography tandem mass spectrometry method, working in a positive electro spray ionization mode (LC-ESI/MS/MS) for the analysis of moxifloxacin and levofloxacin in the serum of multidrug-resistant tuberculosis patients, which was applied on five separate samples for analysis of each analyte [19]. Ultra-high-performance liquid chromatographic (U-HPLC) technique was also applied to determine LEV in human plasma and prostate tissue [21]. For samples from laboratory animals, Christopher J. Destache et al. has described an HPLC-UV method to measure LEV concentration in plasma and CSF samples from a rabbit pneumococcal meningitis model [22]. In addition, Fang et al. has developed a LC-MS/MS method for the high throughput and simultaneous determination of LEV and two anti-tuberculosis drugs (INH and RIF) in mouse plasma and different tissues including brain, lung, liver, kidney and small intestine [23]. A large number of sample preparation techniques were applied: direct injection [12,15]; protein precipitation [6,10–12,14,16,17]; ultrafiltration [7]; liquid–liquid extraction [13]; and solid phase extraction (SPE) [8].

Generally, in contrast to numerous studies to determine LEV in plasma samples, there are only a few publications with a limited number of clinical samples describing the quantification of LEV in human CSF samples [9,18,24,25]. Additionally, to the best of our knowledge, there was only one study [18] has reported the measurement of LEV concentration in both human plasma and CSF samples from TBM patients, using LC/MS and LC/MS/MS.

Therefore, we describe a sensitive and reliable HPLC–FL method, in combination with a SPE process, to determine LEV in a small volume (100 µL) of plasma and CSF from TBM patients. The method can be helpful in both pharmacokinetic studies and routine analysis of LEV in clinical specimens.

### 2. Materials and methods

#### 2.1. Reagents and solutions

All reagents and solvents used were of analytical grade. Potassium dihydrogen phosphate \((\text{KH}_2\text{PO}_4)\), phosphoric acid \((\text{H}_3\text{PO}_4)\), formic acid \((\text{HCO}_2\text{H})\), triethylamine (TEA), HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Water was provided by a Purelab UHQ system (ELGA, Marlow, UK). The reference standards levofloxacin (LEV) and internal standard (IS) enoxacin (ENO) were purchased from Fluka-Sigma Aldrich (Singapore). The different lots of blank human plasma samples from healthy people were supplied by the Blood Transfusion and Haematology Hospital in Ho Chi Minh City. Blank CSF samples were provided by the microbiology laboratory at the Hospital for Tropical Diseases in Ho Chi Minh City as spare aliquots, after routine laboratory investigation, from suspected meningitis patients who had negative diagnosis of bacterial meningitis. CSF blank samples were tested for drug-free LEV before pooling by using the validated method.

#### 2.2. Equipment

The liquid chromatography system was a LaChrom Elite (Merck–Hitachi, Japan) composed by an analyzer, an autosampler L-2200, 2 pumps L-2130, a column Oven L-2350 and a Fluorescence Detector (FD) L-2480. The system was controlled by EZChrom Elite version 3.18 HPLC System Manager Software (Merck–Hitachi, Japan). The analysis was performed on a LichroCart® Purospher Star reversed-phase C8 end-capped column (125 mm × 4 mm i.d., particle size 5 µm; Merck, Darmstadt, Germany), which was equipped with a Lichrospher® (4 mm × 4 mm i.d., particle size 5 µm) guard column (Merck, Darmstadt, Germany). The SPE was performed on Evolute® ABN, 25 mg/1 mL, 96 fixed well plates (Biotage AB, Uppsala, Sweden).

#### 2.3. HPLC analytical conditions

The mobile phase consisted of a mixture of 10 mM KH₂PO₄ and 0.025% of TEA adjusted to pH 3.0 using H₃PO₄ – ACN (88:12, v/v), filtered through 0.20 µm, regenerated cellulose membrane, (Sartorius, Goettingen, Germany) and degassed for 30 min in a sonic bath (AL 04-12, Advantage Lab, Switzerland). The chromatography was performed at 35 °C for seven minutes at a flow rate of 1.2 mL/minute and the autosampler was set at an ambient temperature. The excitation/emission wavelengths (EX/EM) were set to 269/400 nm, 294/500 nm for ENO and LEV respectively. A system suitability test was performed prior to each sequence by injecting six consecutive aqueous standard solutions (3 µg/mL). The tolerated variation was assessed on area response and retention time with an accepted variation of no more than 2%.

#### 2.4. Standard solutions preparation

Stock solutions of LEV (1 mg/mL), ENO (1 mg/mL) were prepared by dissolving the standards in mixture of MeOH and water (1:1, v/v). The different LEV stock solutions were further diluted with formic acid pH 3.0 to obtain fresh working solutions ranging from 0.4 to 400 µg/mL, and 0.4, 1.2, 60 and 300 µg/mL for preparing calibration curve and quality control samples, respectively. ENO/IS solution at concentration of 10 µg/mL was obtained by diluting ENO stock solution (1 mg/mL) in formic acid pH 3.0 solution.

Plasma and CSF calibration curve (CC) and quality control (QC) were prepared by diluting the respective working solutions with blank plasma or CSF with the ratio of 1:20 to give eight CC points at 0, 0.02, 0.1, 0.5, 2.0, 5.0, 10.0, and 20.0 µg/mL. Four QC points were prepared separately in the same way to give the limit of quantification (LOQ), low (QL), medium (QM) and high (QCH) concentrations with 0.02, 0.06, 3.0 and 15.0 µg/mL, respectively.

#### 2.5. Samples preparation

Aliquots of thawed plasma or CSF samples were mixed for 20 s and put in a steam bath at 56 °C for 60 min for the purpose of inactivation to prevent any risk of bacterial or viral infection [26,27]. After cooling down on the bench for 10 min, 300 µL of internal standard/ENO solution (10 µg/mL in formic acid, pH 3.0) were added to 100 µL of sample. The resulting mixture was then vortex mixed for 15 s and rested for two minutes. Finally, the mixture was centrifuged at 9600 × g for five minutes at room temperature.

The supernatant was loaded into Isolute® C18 50 mg or Evolute® ABN 25 mg, 96 fixed well plates (pre-treated with 2 mL of MeOH and 1 mL of formic acid pH 3.0 solution). The SPE plate was washed with 1 mL of formic acid pH 3.0 solution, 1 mL formic acid pH 3.0 solution-MeOH (97:3, v/v) and then dried for two minutes. LEV and ENO (IS) were next eluted with 600 µL of formic acid pH 3.0 solution-MeOH (60:40, v/v) into a 96-collection plate. And then we injected 30 µL of each eluate into the equilibrated chromatographic system.
2.6 Bioanalytical method validation

A complete method validation of LEV in human plasma and CSF was carried out for specificity, sensitivity, linearity, recovery, precision, accuracy, and stability in accordance with USA Food and Drug Administration (FDA) bioanalytical method validation guidelines [28].

2.6.1. Specificity, sensitivity and carryover

The specificity was performed at the LOQ and tested the ability of the method to differentiate the analytes (LEV and IS) in the presence of endogenous interferences of plasma and CSF from six different donors. Current medications, such as anti-tuberculcous drugs (INH, PZA, RIF); antiretroviral drugs (lamivudine, stavudine, zidovudine, nevirapine, lopinavir, ritonavir, efavirenz); and some fluoroquinolones (moxifloxacin, ciprofloxacin), which are likely given to TBM patients, were tested by injecting standard solutions of these compounds at a concentration of 10 μg/mL.

The sensitivity was defined by the limit of detection (LOD) and the limit of quantification (LOQ). The LOD was based on a signal to noise ratio of 3:1. The LOQ was determined a signal to noise ratio of ≥5:1 and at which the precision and accuracy were ≤20% from the determination of six plasma and CSF replicates.

Carryover was evaluated by injecting blank sample following the injection of the upper limit of quantification (ULOQ) samples.

2.6.2. Linearity

The linearity of calibration was determined in the range of 0.02–20 μg/mL and consisted of eight calibration standards including blank sample. The calibration curve was determined by plotting the peak-area ratios (LEV/IS) versus the LEV concentrations using least-squares regression analysis. The best regression model was chosen by evaluating the different regression models, with and without data transformations, as well as different weighting, using model options in EZChrom Elite version 3.18 HPLC system manager software (Merck-Hitachi, Japan) as proposed in the previous publication [29]. Back calculations were performed to quantify the concentrations of LEV in the QC validation sets and clinical samples.

2.6.3. Recovery, precision and accuracy

The recovery yields for LEV using the four QC samples (LOQ, QCL, QCM and QCH, n = 5), and ENO (IS) at 10 μg/mL were calculated by comparing the area response of plasma and CSF spiked samples, processed as described in Section 2.5, to those of unprocessed aqueous solutions at the same concentration levels.

Intra-assay precision and accuracy were determined using five different replicates of LOQ, QCL, QCM and QCH analyzed within the same day. Inter-assay precision and accuracy for plasma and CSF samples were estimated by analyzing five replicates of QC sets on four consecutive days.

Intra-assay, inter-assay and total-assay precision was expressed as the mean relative standard deviation (RSD%) of the concentration area response (LEV/IS), and was determined by using analysis of variance (ANOVA). Accuracy (%) was calculated as [estimated concentration/nominal value] × 100. The variation of the back calculated concentrations from the nominal concentrations should not vary more than 15% for the precision and range from 85 to 115% for the accuracy [28].

2.6.4. Samples dilution effect

Dilution effect was tested to validate the quantification of higher concentrations of analytes, which could be above the ULOQ and encountered during the analysis of clinical samples. Plasma samples (n = 6) were prepared at a LEV concentration of 40 μg/mL, double of the ULOQ, and then diluted five times with blank human plasma. The spiked samples were processed as described in Sections 2.3 and 2.5, while the precision and accuracy of diluted plasma samples is explained in Section 2.6.3.

2.6.5. Stability

The stability study was carried out to experiment the stability of LEV under different conditions. All the stability experiments were evaluated by comparing the concentration area response (LEV/IS) of five different replicates of QCL and QCH against freshly prepared spiked samples. Spiked samples were considered stable if the deviation from the nominal values was within ±15% [28]. The stock solutions of LEV and IS were tested for short term stability at room temperature (RT) for 24 h and for long term stability at −20 °C for a month. Heat inactivation stability was examined by comparing similar aliquots of heated at 56 °C for 60 min versus non-heated samples. The room temperature (RT) stability of plasma and CSF samples was assessed for 24 h. Auto-sampler stability was performed on SPE eluates stored for 24 h at room temperature, corresponding to the time between the first and the last sample analyzed on a 96 well collection plate. Freeze-thaw stability was studied using three consecutive cycles of freezing (−80 °C for at least 24 h) followed by gentle thawing at RT. Long term stability of the spiked samples was examined after one, three and six months storage at −80 °C.

2.7. Method application

2.7.1. Clinical samples from TBM patients

The samples were collected from patients enrolled in the trial “Intensified treatment with high dose rifampicin and levofloxacin compared to standard treatment for adult patients with tuberculous meningitis (TBM-IT): protocol for a randomized controlled trial” (ISRCTN61649292) [3], conducted at the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. For intensive pharmacokinetics analysis, two groups of TBM patients, one with HIV (HIV +) and one without HIV (HIV−) received multiple oral doses of levofloxacin (20 mg/kg twice-daily) in the first eight weeks of treatment at the same time as receiving anti-TB drugs and antiretroviral drugs (HIV+ group). Nine blood samples were collected on the same day, which was selected between day 14 and day 21 of the treatment at different time points: 0, 0.5, 1, 2, 3, 4, 6, 8 and 12 h.

For population pharmacokinetics analysis, on each sample collection day, one randomly time blood and CSF sample were collected on day 28 and day 56 for each patient. For all blood samples, heparinized tubes were used, and plasma was obtained by centrifugation at 2000 × g for 10 min at room temperature. For CSF samples, normal tubes without coagulant were used. All the samples were frozen immediately at −80 °C until assayed.

The clinical samples were analyzed and validated against freshly prepared CC and QC in either blank plasma or CSF. The acceptance criteria on the validation QC sets was <15% for precision and between 85 and 115% for accuracy [28].

2.7.2. Pharmacokinetic analysis

Intensive pharmacokinetic analysis of plasma LEV concentration versus time data was conducted by non-compartmental analysis (with linear trapezoidal method) using PKSolver 2.0 [30]. The pharmacokinetic parameters of LEV determined after oral administration of LEV 500 mg are: half-life (t1/2); steady-state maximum plasma concentration (Cmax,ss); time to reach the Cmax,ss (Tmax); area under the concentration-time curve during the observational period (AUC0–∞); extrapolation to infinity of area under the concentration-time curve (AUC0–∞).

3. Results and discussions

3.1. Method development

In the development of chromatographic conditions for ENO and LEV, we tried to apply different columns: CN, C8 and C18 with a range of mobile phase and pH values. Over pH range of 3–5, LEV and ENO were retained on the column when using phosphate buffer with
acetonitrile. A mobile phase consisting of the buffer at pH 3.0 and ACN (88:12, v/v), and a Purosphere STAR reversed-phase C8 end-capped column provided the conditions, which allowed us to obtain satisfactory separation between ENO and LEV in a reasonable run time. Peak shapes were asymmetrical and slightly tailing at a pH 3.0 using 10 mM KH₂PO₄, but were optimized using 0.025% trimethylamine as a modifier.

Fluorescence detection was used to detect ENO and LEV because of its specificity and greater sensitivity compared to UV or DAD. A time-programmed fluorescence detector was applied in this assay as it can be programmed to change both excitation and emission wavelengths for detection of analytes during separation, which allows us to maintain optimum selectivity and sensitivity throughout the entire chromatogram [31]. This approach was not described in previous publications by using FL for LEV quantification or other fluoroquinolones in biological fluids. The single set of EX/EM were generally set to 295/440 nm, 300/450 nm, 260/455 nm and 310/467 nm for LEV and other FQ [13,14,16,17]. In addition, some publications described HPLC-FL using EX/EM of 293/500 nm or 296/504 nm for LEV and moxifloxacin [10,12]. However, a single pair of EX/EM does not allow optimizing detection of all FQ and an optimum response of FQ was only achieved at an unique EX/EM [32]. According to our experiment, the most sensitive EX/EM were obtained at 269/400 nm and at 294/500 nm for ENO and LEV, respectively; and the maximum intensities of analytes (ENO/IS and LEV) were accordingly achieved. Overall, the fluorescence response of LEV increased from 72.21% to 99.13% compared to those obtained when a single pair of EX/EM were set at 295/440 nm and 310/467 nm; and the LEV intensity was slightly decreased (1.16%) when EX/EM was set at 294/505 nm. For ENO, the improvement of fluorescence response from 76.2 to 87.6% was achieved. The selection of optimum EX/EM for LEV and ENO helped the method achieved the LOQ of LEV lower than previous publications [13,14,16,17]. Fig. 1 showed the chromatogram of different EX/EM for optimizing ENO and LEV detection.

In developing the solid phase extraction procedure for plasma and CSF samples, two kinds of sorbent (C18 and ABN) were used to process the samples and ABN was chosen because it is water-wettable polymeric reversed-phase sorbent that exhibits both hydrophilic and hydrophobic characteristics. The hydrophilic properties of the ABN sorbent obviated the need for maintaining moisture encountered with C18 packing, and consequently, reproducibility and high recovery were achieved. The developed method provided a good chromatographic resolution and specificity. ENO (IS) and LEV were successfully separated from matrix contaminants with the good symmetrical peak shapes at the respective retention time of 4.2 min (ENO/IS) and 5.2 min (LEV) in both plasma and CSF samples (Fig. 2).

3.2. Bioanalytical method validation

3.2.1. Specificity, sensitivity and carryover

None of the concomitant medications (anti-tuberculous drugs, antiretroviral drugs, and some quinolones) interfered with the retention times of ENO and LEV. No interfering peaks were observed in the drug-free human plasma and CSF samples following sample pretreatment procedure, using Evolute® ABN 25 mg 96 fixed well plate. This was also confirmed throughout the entire analysis of clinical samples. No carryover was found in blank sample after injection of ULOQ.

For 100 µL samples, the LOD and LOQ of LEV were 10 ng/mL and 20 ng/mL respectively. There was no difference in the value of LOD and LOQ between plasma and CSF samples. Generally, the LOQ achieved with our method was more sensitive than those reported in previous publications [6-8,10-15,21,23]. This method was successfully achieved with a small sample volume of 100 µL instead of the 200 µL [10,11,14,16,17], 300 µL [21], and 500 µL [7,8] used in previous studies. When LEV analysis was performed by using LC/MS/MS, the LOQ was 10 ng/mL for LEV in human CSF sample [18]. In addition, Sung Joong Lee et al. developed the LC-ESI-MS/MS method for analysis of LEV in 50 µL of serum and achieved a very high sensitivity with a LOQ of 0.13 ng/mL [19], which was more sensitive than that reported in this paper. However, those methods required highly complicated and expensive instruments, which are not always available in the modest laboratories found in developing countries.

3.2.2. Linearity

The eight-point linear calibration curve including blank sample was set up with a range of 0.02–20 µg/mL. The wide range can cover the entire LEV concentrations determined in clinical samples from TBM patients. This was confirmed throughout the entire clinical sample analysis. The calibration curve was determined by plotting the peak-area ratios (ENO/IS and LEV) versus the LEV concentrations using regression analysis. A total of 20 different linear and quadratic regression models, both with and without data transformations, and both with and different weighting: no weight, 1/X, 1/X², 1/Y and 1/Y², were evaluated for LEV quantification in plasma using data obtained during the validation of the assays (1000-fold concentration range and five sets of three QCs on four consecutive days). The best model was linear, log-log transformed, and non-weighting regression model. The optimal model provided a small and equally distributed residual error as well as high coefficient of regression (r² > 0.99) for all LEV calibration curves in both plasma and CSF.

3.2.3. Recovery, accuracy and precision

The mean recoveries of LEV spiked into human plasma and CSF samples in all four QC levels were between 86.41% and 94.69% for plasma, and from 91.26% to 96.47% for CSF. These results indicated a high recovery of LEV from both plasma and CSF via our sample preparation procedure. The mean recovery of the IS, calculated across all validation batches, was 84.83% in plasma and 89.15% in CSF. The intra and inter-assay relative standard deviation were always less than 7% across all QC ranges of LEV in plasma and CSF samples. The accuracies ranged from 96.75% to 101.9% and from 93.00% to 98.67% at all QC levels for LEV in plasma and CSF, respectively. The results showed that the method was accurate and precise for the quantification of LEV in human plasma and CSF samples. The detailed results of the assay are presented in Table 1.

Fig. 1. The chromatograms of different excitation/emission wavelengths for optimizing ENO and LEV detection. (A) Different EX/EM wavelengths for LEV detection: < 1 > 295/440 nm, < 2 > 260/455 nm, < 3 > 310/467 nm, < 4 > 295/504 nm. (B) Two specific EX/EM wavelengths compared to one EX/EM wavelength for detection both ENO and LEV: < 5 > 295/440 nm, < 6 > 260/455 nm, < 7 > 269/400 nm and < 8 > 294/500 nm.

Experimental conditions: Solution contained ENO (10 µg/mL) and LEV (2 µg/mL). HPLC...
plasma sample required to be diluted with matrix because the LEV sub-study of the randomized controlled trial. There was only one 232 TBM patients who were enrolled in the intensive and population PK concentrations in 826 plasma and 477 CSF samples collected from the 3.3. Application of clinical samples from TBM patients

Table 2.

| Conditions | Plasma (n = 5) | CSF (n = 5) |
|------------|---------------|-------------|
|            | QCL (0.06 μg/mL) % (RSD) | QCH (15 μg/mL) % (RSD) | QCL (0.06 μg/mL) % (RSD) | QCH (15 μg/mL) % (RSD) |
| RT-24H     | 95.56 (6.94)  | 96.40 (2.06) | 99.37 (1.40) | 101.1 (1.26) |
| EXT-24H    | 100.6 (1.78) | 100.1 (0.19) | 101.3 (1.37) | 101.2 (1.0)  |
| Heat 56 °C for 60 min | 95.53 (6.34) | 96.08 (2.34) | 99.37 (2.39) | 100.9 (1.07) |
| C3         | 96.80 (7.87) | 97.09 (2.44) | 97.19 (2.88) | 98.84 (1.54) |
| M1 (–80 °C) | 94.33 (6.75) | 98.00 (4.05) | 100.9 (3.22) | 99.10 (5.22) |
| M3 (–80 °C) | 96.37 (6.49) | 97.44 (1.87) | 99.39 (3.62) | 98.65 (2.05) |
| M6 (–80 °C) | 97.57 (6.14) | 101.6 (4.35) | 94.08 (3.14) | 97.64 (3.51) |

Results in % recovery = [mean value in stability sample/mean value in reference] × 100 and RSD: Relative Standard Deviation (%).

3.2.4. Samples dilution effect

For the dilution effect on plasma samples above the ULOQ, the measured concentrations of LEV following a 5-fold dilution was 102.9 ± 0.9 (% ± SD) and the precision was 2.69%. The result demonstrated that dilution integrity with blank plasma didn’t affect the analytical results and it can be applied with confidence.

3.2.5. Stability

The stock solutions of LEV and IS were stable at room temperature for 24 h and at −20 °C for one month. The result of heat inactivation at 56 °C for 60 min revealed no significant degradation in processed plasma and CSF samples; the mean recovery of QCL was 95.53% and 99.37%, and QCH was 96.08% and 100.9% in plasma and CSF samples, respectively. Moreover, the SPE eluates from spiked samples at low and high QC concentrations (n = 5), kept at ambient temperature in the auto-sampler, were stable for 24 h so we can analyze a full 96 well plate without any degradation. Results from the study also showed that the plasma and CSF samples containing LEV at QCL (0.06 μg/mL) and QCH (15 μg/mL) levels remained stable after three consecutive freeze-thaw cycles and after storage in a freezer at −80 °C for one, three and six months.

No significant degradation of LEV was observed under any of the tested storage conditions. The variation of LEV concentration in the described storage conditions ranged from 94.33% to 100.6% and from 94.08% to 101.3% for QCL and from 96.08% to 101.6% and from 97.64% to 101.2% for QCH, in plasma and CSF, respectively. The results of LEV stability in plasma and CSF samples are presented in Table 2.

3.3. Application of clinical samples from TBM patients

The validated method was successfully applied to measure the LEV concentrations in 826 plasma and 477 CSF samples collected from the 232 TBM patients who were enrolled in the intensive and population PK sub-study of the randomized controlled trial. There was only one plasma sample required to be diluted with matrix because the LEV concentration (20.32 μg/mL) was slightly over the upper limit of quantification. The sample was diluted to fall within the calibration range and re-analyzed successfully. The results displayed the excellent clinical applicability for the developed and validated method.

In this paper, we provide the preliminary results of intensive pharmacokinetics analysis at steady state from 14 TBM patients, and we also reported the concentration profile of LEV in plasma and CSF in 20 TBM patients after the levofloxacin treatment regimens on day 28, 56 to demonstrate proof-of-principle that the method works on patient plasma and CSF. In general, there was a large inter-individual variance in LEV pharmacokinetic parameters between, and even within, HIV- and HIV+ groups. The average levofloxacin plasma Cmax,ss was 10.09 μg/mL at 3.86 h and 11.53 μg/mL at 3.0 h after oral administration of LEV (20 mg/kg) for the group of TBM HIV- and TBM HIV+ patients, respectively. The levofloxacin plasma concentration – time profiles of two groups (HIV- and HIV+; n = 7) and the concentration of LEV in plasma and CSF in 20 TBM patients on day 28, 56 after oral
administration with doses of 20 mg/kg are shown in Figs. 3 and 4, respectively. The LEV pharmacokinetic parameters are summarized in Table 3.

The full pharmacokinetic analysis results of LEV in TBM patients, as well as comparison of the AUCLEV in CSF to AUCLEV in plasma ratio, will be described and discussed in detail in a further paper.

4. Conclusions

The HPLC-FL method described here enables an accurate and highly selective quantification of LEV in human plasma and CSF samples. The method requires only 100 μL of plasma or CSF to give 10 ng/mL LOD and 20 ng/mL LOQ. This is of significant benefit for clinical studies, where volumes of plasma and CSF are often limited. Moreover, as the total time of chromatography analysis is only seven minutes, this method could be a potential tool for real-time pharmacokinetic and drug monitoring studies of LEV. We have successfully applied this method to analyze LEV in plasma and CSF samples from 232 Vietnamese TBM patients and the preliminary results of intensive pharmacokinetic analysis of LEV from 14 patients were described using this method.

Author contributions

GT, TTH, TP, TTHC, DH, PVT contributed to the conception of the study and the experimental design. TTHC, PVT, NDKT, PNP managed all participants and collected samples. PVT, TP, NDKT, PNP developed and validated the HPLC quantification method. PVT, NDKT analyzed clinical samples and data. PVT, NDKT, TP wrote the paper. NTTT: Head of TB group, OUCRU Vietnam provided a significant support to perform assays and contributed to the writing of this article. All authors have reviewed and/or edited the drafts of the manuscript, approve and take responsibility for the content of final submitted version.

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