Journal of Analytical Science and Technology

RESEARCH ARTICLE Open Access

Development and validation of an improved HPLC-UV method for simultaneous determination of lamotrigine and oxcarbazepine and its active metabolite 10,11-dihydro-10-hydroxycarbazepine in human blood plasma and comparison with an UHPLC-MS/MS method

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Abstract

Lamotrigine (LTG) and oxcarbazepine (OXC) are first-line drugs for epilepsy treatment. Their large pharmacokinetics variabilities and relations between efficacy and toxicity and blood plasma concentration require routine monitoring for dose adjustment. In this study, we developed and validated a simple, accurate, and reliable method for simultaneous determination of LTG, OXC and 10,11-dihydro-10-hydroxycarbazepine (MHD) in human blood plasma by high-performance liquid chromatography-ultraviolet detection (HPLC-UV) with a simple one-step protein precipitation using methanol (1% acetic acid) and 15 min elution time under isocratic elution at 1 mL/min. Calibration range was 2.4 to 120 mg/L for LTG, OXC, and MHD. The intra-day and inter-day bias were − 8.84 to 4.18%, and the imprecision was less than 8.08% for all analytes. The internal standard (fluconazole) normalized recovery was 96.30 to 107.69% for LTG, 98.51 to 111.04% for MHD, and 95.04 to 109.86% for OXC. A total of 186 LTG samples and 25 MHD samples were used to evaluate the agreement between HPLC-UV and ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS/MS) by Passing-Bablok regression and Bland-Altman plot. The mean bias and the 95% limits of agreement (95% LOA) of the two measurements were 0.575 mg/L and − 1.238 to 2.387 mg/L for LTG (n = 186) and − 1.222 mg/L and − 8.271 to 5.827 mg/L for MHD (n = 25), which indicated the UV method was comparable with the MS method for LTG and MHD analysis.

Keywords: HPLC-UV, Lamotrigine, Oxcarbazepine, 10,11-Dihydro-10-hydroxycarbazepine, Method comparison

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Introduction

Drug therapy is the most important treatment for epilepsy control for most of the patients (Palte et al., 2018). New generations of anti-epileptic drugs (AEDs) such as lamotrigine (LTG) and oxcarbazepine (OXC) have gradually replaced traditional AEDs as first-line drugs for their less drug interactions and better toleration (Theitler et al., 2017).

LTG is widely used in monotherapy and combined therapy for epilepsy control, especially for special populations such as children, the elderly, and pregnant women (Italiano & Perucca, 2013; Westley & Morris, 2008). The most important and prevalent toxicity of LTG is toxic epidermal necrosis with a mortality of about 30%, and its incidence increases significantly when LTG plasma level is above 15 mg/L (Alabi et al., 2016; Baldelli et al., 2015a; Pereira et al., 2007). LTG plasma level could be affected by various factors including age, pregnancy, liver and kidney function, smoking, genetic background, and combined medication (Italiano & Perucca, 2013; Petrenaite et al., 2005; Clark et al., 2013; Lovric et al., 2018; Brzakovic et al., 2012; Milosheska et al., 2016). Therefore, LTG dose adjustment is a challenge for clinicians and pharmacist to achieve satisfied efficacy and avoid life-threatening toxicity.

OXC is another first-line drug for the treatment of generalized tonic-clonic and partial seizure (Wellington & Goa, 2001). After oral administration, OXC is rapidly reduced to its active metabolite 10,11-dihydro-10-hydroxycarbazepine (MHD), which is the biomarker for its pharmacological activity (May et al., 2003) and toxicity (Wellington & Goa, 2001; Striano et al., 2006). However, the pharmacokinetics of MHD varied greatly among individuals, and it could be influenced by age, body weight, renal function, and pregnancy (Patsalos et al., 2008; Lin et al., 2018). Based on the characteristics of LTG and OXC mentioned above, therapeutic drug monitoring (TDM) is recommended for safe and effective use of the two drugs (Patsalos et al., 2008).

Previously, several methods were developed and validated for the determination of the three analytes with many limitations. One HPLC-UV method published in 2013 used solid-phase extraction for sample pretreatment, but it was too expensive to afford for developing countries (Serralheiro et al., 2013). Other methods did not simultaneously determine MHD and LTG (EMEA, 2011; Almeida et al., 2002). We also developed and validated an UHPLC-MS/MS method for the simultaneous determination of the three analytes. However, during routine clinical application of the method, we found that an alternative method was required due to the following reasons: immunoassay for LTG, OXC, and MHD measurement is not available in China; for routine monitoring of clinical samples, the LC-MS should be ready during the work day, which resulted in big dissipate of machine-hour; UV method was more affordable for routine monitoring than the LC-MS method, especially in developing countries like China; and during the long-time clinical application, LC-MS might be shut down due to various troubles, however the drug monitoring should not be interrupted to ensure medical quality. This study was designed to develop and validate a simple, accurate, and reliable HPLC-UV method for simultaneous determination of LTG, OXC, and MHD. The agreement between HPLC-UV method and LC-MS/MS method for LTG and MHD analysis was also evaluated by Passing-Bablok regression and Bland-Altman plot.

Method and materials

Standards and reagents

LTG (98% purity) was purchased from HePeng (Shanghai, China) Biotech, Ltd. OXC (Lot, 100657-201102, 99.8% purity) and fluconazole (FCZ, internal standard, IS) (Lot, 100314-201204, 99.8% purity) were acquired from the National Institutes for Food and Drug Control (Beijing, China). MHD (Lot, 20140827, 97% purity) was obtained from Nanjing Chemlin Chemical Industry Company, Ltd. Methanol and acetonitrile were purchased from Fisher Scientific (Waltham, USA). Acetic acid and potassium dihydrogen phosphate were obtained from Sinopharm Chemical Reagent Company, Ltd. Human blank plasma was provided by healthy volunteers and checked to ensure they did not contain LTG, MHD, OXC, or FCZ. Ultrapure water was produced by Millipore ultra-pure water system (Bedford, USA).

Equipment and chromatographic conditions

Experiment was carried out on Dionex UltiMate 3000 (Thermo Fisher, USA) with a PDA3000 UV Detector (Thermo Fisher, USA) equipped with an Acclaim™ C18 column (Thermo, 4.6 × 150 mm, 5 μm particles). Instrument control and data acquisition were performed by the Chromeleon™ software (Thermo Fisher, version 7).

A mixture of potassium dihydrogen phosphate buffer (50 mM) and methanol (61:39) was used for separation at 1.0 mL/min. The column oven was maintained at 37 °C. LTG, OXC, and MHD were monitored at 210 nm.

Preparation of stock and working solutions

Two sets of separately weighted stock solutions (2400 mg/L for LTG, OXC, and MHD) were used to prepare calibration working solutions and quality control (QC) working solutions by dissolving appropriate amount of drugs in methanol-acetonitrile (50:50, v/v). Working solutions for calibration samples were prepared at 12, 24, 60, 150, 300, and 600 mg/L (for LTG, OXC, and MHD). Working solutions for QC samples were prepared at 12, 30, 120, 240, and 480 mg/L (for LTG, OXC, and MHD).

IS stock solution was prepared by dissolving FCZ in methanol at 4400 mg/L, and IS working solution was 440 mg/L. All stock and working solutions were stored at − 80 °C and thawed to room temperature before use.

Preparation of calibration and QC samples

Ten microliters of analytes working solution and 10 μL of IS working solution were added to 50 μL of analytes- and IS-free blood plasma, then 130 μL of methanol (1% acetic acid) was added following a 5-min vortex-mixing and a 2-min centrifugation at 12000×*g* for protein precipitation. Sixty microliters of the supernatant was transferred into a sample vial, and 20 μL was injected for analysis. Thus, calibration samples for curve fitting were prepared at 2.4, 4.8, 12, 30, 60, and 120 mg/L for LTG, MHD, and OXC, and QC samples were prepared at 2.4, 6, 24, 48, and 96 mg/L for LTG, MHD, and OXC. The final concentration was 22 mg/L for IS.

Sample extraction and patient enrollment

This study was approved by the Ethics Committee of Beijing Tiantan Hospital, Capital Medical University, Beijing, Peoples' Republic of China. Informed consent was obtained from all individual participants included in the study. About 1 to 2 mL of venous blood was obtained from patients with epilepsy on OXC and/or LTG therapy. After 5 min centrifugation at 5000×*g*, 50 μL of the blood plasma was transferred and mixed with 10 μL of IS, then 140 μL of methanol (1% acetic acid) was added, and then a 5-min vortex-mixing and a 2-min centrifugation at 12,000×*g* were performed for protein precipitation. Subsequently, 60 μL of the supernatant was transferred into the sample vial, and 20 μL was injected for analysis.

Method validation

Method validation was performed in accordance with the EMEA and FDA guidelines including selectivity, lower limit of quantitation (LLOQ), carry-over, linearity, accuracy, precision, recovery, and stability (FDA, 2013; EMEA, 2011).

Selectivity and LLOQ

The selectivity was assessed by comparing the peak area of analytes- and IS-free plasma from six individuals with those of LLOQ samples. The peak area of interfering peaks in blank plasma should be less than 20% of that of the LLOQ sample for analytes (FDA, 2013; EMEA, 2011). The lowest concentration of the calibration curve (2.4 mg/L for LTG, MHD, and OXC) was defined as the LLOQ. LLOQ is reliable when its bias and precision are within ± 20% and < 20%, respectively.

Linearity and carry-over

Least square method was used for linear regression for all analytes. A weighting factor of $1/x^2$ was chosen for its lower relative error and higher r^2 for linear regression (Almeida et al., 2002). The linear regression equations and correlation coefficients were calculated. Carry-over was evaluated by injecting a blank sample immediately following the highest concentration of the calibration sample. Acceptable carry-over is achieved when the peak area of interfering peaks in blank plasma was less than 20% of those in the LLOQ sample for analytes, and 5% for the IS (FDA, 2013; EMEA, 2011).

Accuracy and precision

The inter-day and intra-day accuracy and precision were estimated by analyzing five replicates of QC samples at 2.4, 6, 24, 48, and 96 mg/L for all analytes on 12 days, and the bias and coefficient of variation (CV%) were calculated. The accuracy was acceptable when bias was within ± 15% (± 20% for LLOQ), and the acceptable precision was obtained when CV was less than 15% (20% for LLOQ).

Recovery

Two batches of QC samples at 2.4, 6, 24, 48, and 96 mg/L for LTG, OXC, and MHD were prepared to evaluate the recovery as follows (FDA, 2013; EMEA, 2011): (A) LTG, OXC, MHD, and IS spiked in blank plasma from six individuals with extraction; (B) LTG, OXC, MHD, and IS in post-extracted blank plasma from the same six individuals. The ratios of $(A_{analyte}/B_{analyte}) \times 100\%$ were defined as recovery while the ratios of $(A_{analyte}/B_{analyte})/(A_{IS}/B_{IS}) \times 100\%$ were defined as IS normalized recovery. The recovery was reliable when IS normalized recovery was consistent at all QC levels.

Stability

Stability was assessed by using four levels of QC samples. The bias between post-stored samples and the QC samples prepared at the same day was used to evaluate the stability of analytes during sample preparation, storage, and analysis: in plasma: 25 °C for 6 h and 9 h, three freeze-thaw cycles from − 80 to 25 °C, and − 80 °C for 15 days; post-extraction: 25 °C for 10 h and 24 h, 4 °C for 24 h, and − 80 °C for 17 days. Analytes were stable when the bias of QC samples was within ± 15%.

The influence of other co-medicated AEDs on the measurement of LTG, OXC, and MHD

The antiepileptic drugs commonly used in China are LTG, OXC, carbamazepine (CBZ), phenobarbital (PB), phenytoin (PHT), levetiracetam, topiramate, ethosuximide, and valproate. The influence of valproic acid, levetiracetam, topiramate, and ethosuximide on the

measurement of these analytes was not evaluated because all of them were lack of ultra-violet absorption. To make sure the commonly used AEDs did not influence the determination of LTG, OXC, and MHD, samples were prepared by adding working solutions (concentration 24 mg/L for LTG, OXC, and MHD) into the plasma obtained from patients taking PB, PHT, or CBZ (the plasma concentrations of these co-medicated AEDs were all within the therapeutic window) and analyzed within a runtime of 30 min and 15 min, respectively. Then, two blank samples (methanol only) were injected immediately.

The UHPLC-MS/MS method

The UHPLC-MS/MS method was performed on an Acquity UHPLC H-Class (Waters, MA, USA) tandem 5500 QTRAP mass system (AB SCIEX, CA, USA). The calibration range was 0.06 to 12 mg/L for LTG and 0.03 to 6 mg/L for MHD using LTG-$^{13}C_3$ and MHD-D_4 as internal standards. The quantitative ion pair was *m/z* 256.0 > 144.9 for LTG, *m/z* 255.1 > 194.1 for MHD, *m/z* 259.0 > 144.9 for LTG-$^{13}C_3$, and *m/z* 259.1 > 198.1 for MHD-D_4. The separation was achieved by using methanol (A, 0.1% formic acid) and water (B, 0.1% formic acid) at 0.4 mL/min under gradient elution with a run time of 3 min. Calibrators at 0.03, 0.06, 0.3, 0.6, 3, and 6 mg/L for MHD and 0.06, 0.12, 0.6, 1.2, 6, and 12 mg/L for LTG and QC samples at 0.09, 0.225, 2.25, and 4.5 mg/L for MHD and 0.18, 0.45, 4.5, and 9 mg/L for LTG were prepared by spiking 10 μL working solution and 10 μL IS into 100 μL drug- and IS-free blood plasma and vortex-mixed and centrifuged with 300 μL methanol for precipitation. The supernatant was diluted 10 times, and then, 1 μL of the supernatant was injected for analysis. Ten-fold dilution by plasma or water did not affect the measurement of analytes in LC-MS/MS method, by doing that, the method could cover the clinical plasma concentration range for all analytes.

Statistical analysis

Statistical analysis was performed by using SPSS (SPSS Inc., Chicago, IL, USA, version 17) and MedCale (MedCale Software bvba, Ostend, Belgium, version 15.8) software. Statistical significance was defined as a *P* value less than 0.05.

Application and comparison

A total of 186 samples for LTG (25 samples for MHD) were analyzed by the HPLC-UV and the UHPLC-MS/MS methods. The equation and the correlation coefficient were evaluated by Passing-Bablok regression (a linear regression procedure with no special assumptions regarding the distribution of the samples and the measurement errors) and Pearson test for both two methods (Passing & Bablok, 1983). The mean value of the two measurements was evaluated by paired samples *t* test or Wilcoxon test. Bland-Altman plot was used to supplement the results of the Passing-Bablok regression and evaluate the agreement of the two methods (Bland & Altman, 1986; Bland & Altman, 1999).

Results

Linearity and LLOQ

Figure 1 shows the typical chromatograms of the HPLC method. The typical linear regression equation is $y = 5.8274 \times x - 0.767$, $r^2 = 0.9983$ for LTG; $y = 4.0106 \times x + 1.278$, $r^2 = 0.9972$ for MHD; and $y = 2.9969 \times x - 0.028$, $r^2 = 0.9967$ for OXC (*x*, analytes concentration; *y*, peak area ratio of analytes to IS). The bias and imprecision of LLOQ samples were − 12.54 to 4.41% and < 11.74% for LTG, − 15.72 to 8.44% and < 18.96% for MHD; − 17.62 to 13.33% and < 15.94% for OXC, respectively (supplementary data-accuracy).

Selectivity and carry-over

The signal-to-noise ratio of LLOQ samples was 17.0, 32.3, and 56.7 for LTG, MHD, and OXC, respectively. Interfering peaks at the elution time of analytes in blank plasma were less than 20% of those at LLOQ samples and IS did not influence the analysis. Carry-over was negligible for all analytes and IS.

Accuracy and precision

Table 1 shows the intra-day and inter-day accuracy and precision of the method. The intra-day and inter-day bias at five QC levels were − 7.99 to 4.18% for LTG, − 8.84 to 2.97% for MHD, and − 5.30 to 3.56% for OXC; the imprecision was < 8.08% for LTG, < 7.22% for MHD, and < 7.79% for OXC.

Recovery

At four QC levels, the recovery was 90.74 to 95.53% for LTG, 92.78 to 99.65% for MHD, and 89.37 to 99.31% for OXC, respectively (Table 2). The IS normalized recovery was 96.30 to 107.69% for LTG, 98.51 to 111.04% for MHD, and 95.04 to 109.86% for OXC, separately. Thus, the extraction recovery was consistent and acceptable.

Stability

LTG, OXC, and MHD were stable under all storing conditions at four QC levels with a bias ranged from − 13.24 to 19.60% (Additional file 1: Appendix 1).

Calibration and QC of the UHPLC-MS/MS method

The calibration curves of LTG and MHD were linear, the intra- and inter-day bias were − 13.11 to 0.27%, and imprecision was all less than 13.32% at four QC levels.

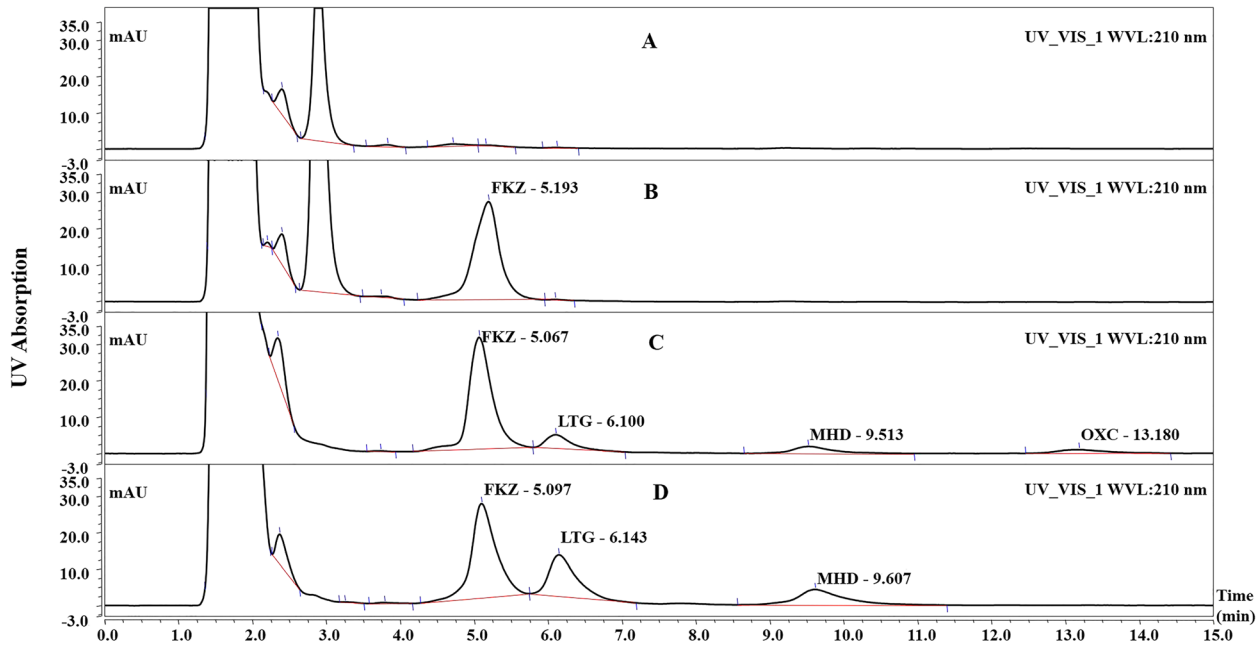

Fig. 1 Typical chromatogram of lamotrigine (LTG), 10,11-dihydro-10-hydroxycarbazepine (MHD), oxcarbazepine (OXC), and internal standard (fluconazole) obtained from: **a** blank plasma, **b** blank plasma only spiked with internal standard (fluconazole), **c** lowest limit of quantitation (2.4 mg/L for LTG, MHD, and OXC), **d** sample from a patient

The influence of other co-medicated AEDs on the measurement of LTG, OXC, and MHD

The influence of PB, PHT, and CBZ on the analysis of LTG, OXC, and MHD is illustrated in Additional file 1: Appendix 2. PB, PHT, and CBZ were well separated from the three analytes with a retention time of 5.20 min for FCZ, 6.22 min for LTG, 7.90 min and 8.61 min for PB, 9.73 min for MHD, 13.40 min for OXC, 21.20 min for PHT, and 26.57 min for CBZ in a 30-min elution (Additional file 1: Appendix 2-A). With a 15-min elution, the retention time of PB was 7.90 and 8.61 min, but PHT and CBZ were retained on the column, and they were eluted from the column in the next 15-min elution with a retention time of 3.72 min and 9.15 min (Additional file 1: Appendix 2-B, C, D).

Method application and comparison

LTG measurements for both two methods were abnormally distributed ($P < 0.05$ for Kolmogorov-Smirnov test), while MHD measurements were normally distributed ($P < 0.2$ for Shapiro-Wilk test). The results of UHPLC-MS/MS method were 4.50 ± 2.66 (0.51 to 22.70) mg/L for LTG and 12.08 ± 7.66 (0.04 to 27.30) mg/L for MHD. The results of HPLC-UV method were 5.61 ± 2.58 (2.42 to 22.57) mg/L for LTG and 11.14 ± 5.88 (3.33 to 25.90) mg/L for MHD. Between the two measurements, the mean value was different ($P < 0.0001$) for LTG, but it was similar for MHD ($P = 0.102$). Evaluated by Passing-Bablok regression (Fig. 2a), the regression equation of the two measurements was HPLC-UV = 0.960 × UHPLC-MS/MS + 0.64 ($r = 0.936$) for LTG and HPLC-UV = 0.887 × UHPLC-MS/MS + 1.23 ($r = 0.845$) for MHD.

There was no correlation between the difference and magnitude in both two measurements for both LTG and MHD; therefore, comparison was achieved by using the initial data of both measurements (Bland & Altman, 1986; Bland & Altman, 1999). The mean bias and the 95% LOA of the two measurements were 0.575 mg/L and − 1.238 to 2.387 mg/L for LTG, and − 1.222 mg/L and − 8.271 to 5.827 mg/L for MHD. Eight LTG (8/186, 4.30%) and three MHD samples (3/25, 12%) fallen outside the range of their 95% LOA (Fig. 2b), respectively.

Discussion

Optimization of chromatographic conditions

In published studies, 210 nm and 270 nm were commonly used wavelength for the measurement of LTG, MHD, and OXC, and their maximum UV absorptions were 210 nm, 230 nm, and 255 nm, respectively (Baldelli et al., 2015a; Greiner-Sosanko et al., 2007; Contin et al., 2010; Fortuna et al., 2010). When 270 nm was used for detection, the absorption was 7.22 times lower than those at 210 nm for LTG at LLOQ, and MHD and OXC were undetectable at LLOQ (Additional file 1: Appendix 3). We found that all analytes had a relatively high absorption at 210 nm, which was chosen for detection (Additional file 1: Appendix 4). Methanol and

Table 1 The precision and accuracy of LTG, MHD, and OXC in human blood plasma (mean ± standard deviation)

Measurement	Drug	Analyte concentration (mg/L)	Measured concentration (mg/L)	Bias (%)	Coefficient variation (%)
Intra-day, $n = 5$	LTG	96	100.015 ± 8.078	4.18	8.08
		48	46.138 ± 2.457	− 3.88	5.32
		24	22.082 ± 0.789	− 7.99	3.57
		6	5.703 ± 0.427	− 4.95	7.49
		2.4	2.308 ± 0.08	− 3.84	3.45
	MHD	96	98.85 ± 7.1	2.97	7.18
		48	46.773 ± 2.372	− 2.56	5.07
		24	22.536 ± 0.788	− 6.10	3.49
		6	5.89 ± 0.425	− 1.83	7.22
		2.4	2.188 ± 0.102	− 8.84	4.67
	OXC	96	99.414 ± 7.201	3.56	7.24
		48	47.06 ± 2.307	− 1.96	4.90
		24	22.729 ± 0.794	− 5.30	3.49
		6	5.953 ± 0.379	− 0.78	6.37
		2.4	2.345 ± 0.183	− 2.30	7.79
Inter-day, $n = 60$	LTG	96	98.769 ± 2.952	2.88	2.99
		48	46.561 ± 1.076	− 3.00	2.31
		24	23.337 ± 0.491	− 2.76	2.10
		6	5.878 ± 0.091	− 2.03	1.55
		2.4	2.337 ± 0.084	− 2.63	3.58
	MHD	96	96.934 ± 2.884	0.97	2.97
		48	46.565 ± 1.416	− 2.99	3.04
		24	23.468 ± 0.475	− 2.22	2.03
		6	6.053 ± 0.128	0.89	2.12
		2.4	2.335 ± 0.117	− 2.69	5.01
	OXC	96	98.103 ± 2.908	2.19	2.96
		48	46.931 ± 1.074	− 2.23	2.29
		24	23.912 ± 0.473	− 0.37	1.98
		6	6.114 ± 0.204	1.89	3.34
		2.4	2.336 ± 0.066	− 2.68	2.83

Abbreviations: *LTG* lamotrigine, *MHD* 10,11-dihydro-10-hydroxycarbazepine, *OXC* oxcarbazepine

acetonitrile were optimized for separation. Acetonitrile was tested at first, and we found that it would greatly shorten the retention time of LTG to 2.62 min and its quantification will be affected by protein peaks (Additional file 1: Appendix 5-A). After decreasing the proportion of acetonitrile (15% acetonitrile-water) to improve LTG retention (6.69 min), MHD and OXC were eluted out of 15 min, which was too long to use in clinical monitoring (Additional file 1: Appendix 5-B). This problem was solved when we used methanol (Additional file 1: Appendix 3-A). Several studies have shown that isocratic elution by methanol-water was useful for separation, and we also used it for optimization for its low price (Westley & Morris, 2008; Rivas et al., 2010). Thus, isocratic elution by methanol-water was used for separation. The use of potassium dihydrogen phosphate in water (20–100 mM) could increase the intensity and shorten the retention time of three analytes (Additional file 1: Appendix 6) (Greiner-Sosanko et al., 2007; Contin et al., 2010; Rivas et al., 2010; Kimiskidis et al., 2007; Zhang et al., 2019). In summary, isocratic elution with methanol to water-50 mM potassium dihydrogen phosphate at 39:61 was used for separation, and 210 nm was used for detection to get acceptable peak shape, sensitivity, and run time.

Different precipitants were tested. Acetic acid was useful to separate PB and MHD (Jebabli et al., 2015) as well as to improve peak shape for three analytes (Additional file 1: Appendix 7). Moreover, by using acetic acid as precipitant, a smaller precipitant volume (3 to 0.6 times

Table 2 The recovery of LTG, MHD, and OXC in human blood plasma (mean ± standard deviation, $n = 6$)

Drug	Analyte concentration (mg/L)	Recovery-analytes (%)	IS normalized recovery (%)
LTG	96	96.416 ± 11.993	107.685 ± 19.554
	48	95.532 ± 10.998	104.92 ± 19.041
	24	94.036 ± 6.047	101.893 ± 8.805
	6	90.742 ± 10.653	96.295 ± 1.431
MHD	96	99.648 ± 12.17	111.04 ± 18.242
	48	98.922 ± 10.127	108.393 ± 16.871
	24	95.792 ± 6.056	103.762 ± 8.332
	6	92.784 ± 10.517	98.514 ± 2.574
OXC	96	97.591 ± 12.581	108.75 ± 18.517
	48	96.473 ± 9.032	105.696 ± 15.734
	24	95.643 ± 6.356	103.66 ± 9.514
	6	89.374 ± 9.427	95.038 ± 5.174
FCZ (IS)	96	90.983 ± 13.712	-
	48	92.412 ± 11.783	
	24	92.782 ± 9.522	
	6	94.309 ± 11.685	

Abbreviations: LTG lamotrigine, *MHD* 10,11-dihydro-10-hydroxycarbazepine, *OXC* oxcarbazepine, *IS* internal standard (fluconazole), *CV* coefficient of variation

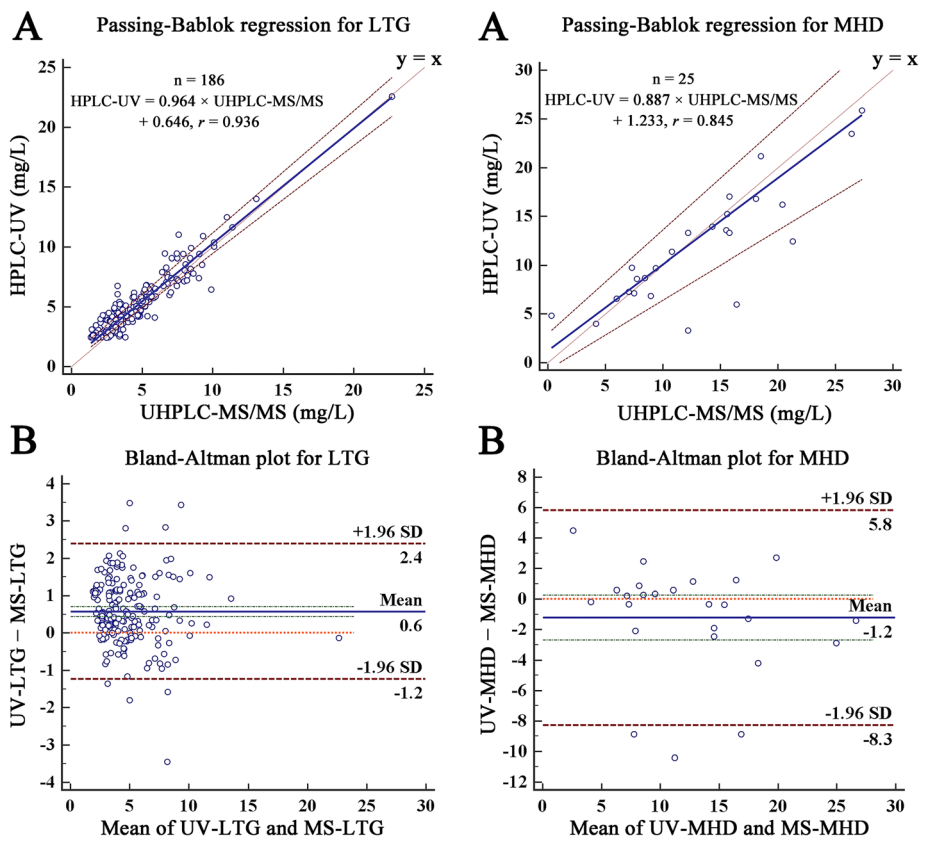

Fig. 2 Passing-Bablok regression and Bland-Altman plot of UHPLC-MS/MS and HPLC-UV methods in the analysis of LTG and MHD in human plasma. **a** Passing-Bablok regression. **b** Bland-Altman plot. The green line in **b** was 95% confidence interval of the mean bias

of plasma volume) and a higher response of analytes were obtained compared to methanol or acetonitrile. However, incomplete protein precipitation was observed, and the response of protein peak treated with acetic acid was 3.5-fold higher than those with methanol. Moreover, white flocculent precipitates appeared in acetic acid-precipitated samples after storing at room temperature for 2 h. Although we could solve this problem by storing the post-precipitated sample at 4 °C for 30 min, the time-consuming precipitation was not suitable for its clinical application. Due to the benefits of acetic acid for protein precipitation, different concentrations of acetic acid (0.1%, 0.3%, 1%, 3%) were added in methanol for sample pretreatment. The results indicated that methanol with 1% acetic acid had the highest intensity and acceptable peak shapes (Additional file 1: Appendix 8), and this simple and effective method was chosen for protein precipitation and extraction (Serralheiro et al., 2013; Jebabli et al., 2015). The low proportion of methanol (39%) for separation could result in column pollution; therefore, column should be washed every day after analysis to guarantee the column efficiency.

PB and PHT would not affect the determination of LTG, MHD, and OXC because they were well separated from the three analytes. However, CBZ might interfere MHD analysis in the next injection (9.15 min for CBZ and 9.73 min for MHD). Therefore, a blank sample should be injected immediately after the analysis of samples containing CBZ to avoid its influence on MHD measurement.

Method validation

The linear range was 2.4–120 mg/L for all analytes, and each of them could meet their therapeutic ranges (2.5–15 mg/L for LTG and 3–35 mg/L for MHD) (Patsalos et al., 2008). The IS normalized recovery (96.30 to 107.69% for LTG, 98.51 to 111.04% for MHD, and 95.04 to 108.75% for OXC at four QC levels) was similar compared with the values in many published studies (84.16 to 103.7% for LTG, 77.30 to 104.40% for MHD, and 79.30 to 96.93% for OXC) (Serralheiro et al., 2013; Kimiskidis et al., 2007; Begas et al., 2017; Alves et al., 2007; Contin et al., 2005). The freeze-thaw and long-term storing stabilities were similar to the published results (Serralheiro et al., 2013; Baldelli et al., 2015b; Morgan et al., 2011; Antonilli et al., 2011). Three analytes were stable at room temperature for 9 h, while in the literature, they were stable for 4 h (Serralheiro et al., 2013; Fortuna et al., 2010).

In actual daily measurements, it is not convenient to obtain large amount of drug-free human plasma. Therefore, we tried to use water instead of plasma as matrix for the preparation of calibration and QC samples. The results indicated that bias between water and plasma samples at five QC levels (2.4 to 96 mg/L) was − 14.89 to 11.16% (supplementary data-serum-water), which indicated that water could be used to replace plasma.

Method application and comparison

A good correlation (r = 0.936) between HPLC-UV and UHPLC-MS/MS for LTG analysis was observed, and the correlation equation was almost coincident with the line of equality ($y = x$) (Bland & Altman, 1986; Bland & Altman, 1999). The sample size was big enough as suggested (> 100) to get a stable result for LTG but not for MHD (Bland, 2004). HPLC-UV was higher than UHPLC-MS/MS for LTG analysis with a mean value of 0.6 mg/L, which could be explained by the co-elution of endogenous impurities attributed to the well-known less specificity of UV detector (Zhang et al., 2019). Quantitative microsphere system LTG immunoassay was applied in the clinical practice and compared with HPLC-UV method in the previous studies (Westley & Morris, 2008; Baldelli et al., 2015a; Morgan et al., 2011). The results demonstrated a significant overestimation (6 to 21%) for the immunoassay which might be caused by the cross-reaction of the antibody used in the assay with the N2-glucuronide metabolite of LTG (Westley & Morris, 2008; Morgan et al., 2011). The mean bias and 95% LOA of MHD observed between the two methods were relatively high perhaps because of the small sample size. Due to lack of clinical information, we did not know whether this degree of variation for drug measurement will affect clinical therapy.

Conclusion

A simple, accurate, and reliable method for simultaneous determination of LTG, OXC, and MHD by HPLC-UV was developed, validated, and applied in clinical practice. The experiences in method development and validation might be useful for other researchers. The 95% LOA of HPLC-UV and UHPLC-MS/MS was − 1.238 to 2.387 mg/L for LTG and − 8.271 to 5.827 mg/L for MHD. More clinical information was required to evaluate their clinical agreements.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40543-019-0198-9.

Additional file 1: Appendix 1. The stability of LTG, MHD, and OXC in human blood plasma (mean ± standard deviation, n = 5); **Appendix 2**, The chromatogram of FCZ, LTG, PB, MHD, OXC, PHT, and CBZ under the isocratic elution of 39% methanol-water (50 mM potassium dihydrogen phosphate) within the total run time 32 min; The chromatogram of FCZ, LTG, PB, MHD, OXC, PHT, and CBZ under the isocratic elution of 39% methanol-water (50 mM potassium dihydrogen phosphate) in one injection of analytes and followed by two injections of methanol within the run time 15 min respectively; **Appendix 3**, The chromatogram of FCZ, LTG, MHD, and OXC at LLOQ detected at: (A) 210 nm; (B) 270 nm;

Appendix 4, The UV absorption spectrum of: (A) LTG; (B) MHD; (C) OXC; **Appendix 5**, The chromatogram of LTG, MHD, and OXC under the isocratic elution of: (A) 30% acetonitrile-water; (B) 15% acetonitrile-water; **Appendix 6**, The chromatogram of LTG, MHD, and OXC under the isocratic elution of: (A) 45% methanol-water; (B) 45% methanol-water (50 mM potassium dihydrogen phosphate); the retention time of LTG, MHD, and OXC was: (A) 4.69, 6.08, and 7.90 min, respectively; (B) 3.94, 5.19, and 6.59 min, respectively; the UV absorption of three analytes was: (A) 1.99, 2.00, and 1.59 mAU*min, respectively; (B) 3.32, 2.06, and 1.72 mAU*min, respectively; **Appendix 7**, The chromatogram of FCZ, LTG, MHD, and OXC under the isocratic elution of 40% methanol-water (50 mM potassium dihydrogen phosphate) with the use of: (A) 10% acetic acid; (B) methanol as the precipitant; **Appendix 8**, The chromatogram of FCZ, LTG, MHD, and OXC under the isocratic elution of 39% methanol-water (50 mM potassium dihydrogen phosphate) with the use of: (A) methanol (0.3% acetic acid); (B) methanol (0.1% acetic acid); (C) methanol (1% acetic acid); (D) methanol (3% acetic acid); (E) acetonitrile (0.3% acetic acid) as the precipitant; The peak area of FCZ, LTG, MHD, and OXC with the use of: (A) methanol (0.3% acetic acid); (B) methanol (0.1% acetic acid); (C) methanol (1% acetic acid); (D) methanol (3% acetic acid); (E) acetonitrile (0.3% acetic acid) as the precipitant.

Abbreviations

95% LOA: 95% limits of agreement; AEDs: Anti-epileptic drugs; CBZ: Carbamazepine; FCZ: Fluconazole; HPLC-UV: High-performance liquid chromatography-ultraviolet detection; IS: Internal standard; LLOQ: Lower limit of quantitation; LTG: Lamotrigine; MHD: 10,11-Dihydro-10-hydroxycarbazepine; OXC: Oxcarbazepine; PB: Phenobarbital; PHT: Phenytoin; QC: Quality control; TDM: Therapeutic drug monitoring; UHPLC-MS/MS: Ultra-performance liquid chromatography-mass spectrometry

Acknowledgements

Not applicable

Authors' contributions

JSY and ZQ analyzed and interpreted the method validation data and were a major contributor in writing the manuscript. ZDJ finished the acquisition and analysis. MSH and ZZG drafted the work or substantively revised it. All authors read and approved the final manuscript.

Funding

This study was supported by the Beijing Municipal Administration of Hospitals (ZYLX201827).

Availability of data and materials

All data generated or analyzed during this study are included in this published article (Additional file 1).

Competing interests

The authors declare that they have no competing interests.

Received: 8 August 2019 Accepted: 14 November 2019
Published online: 18 December 2019

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