Talanta 158 (2016) 77–88

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development, validation and clinical application of an online-SPE-LC-HRMS/MS for simultaneous quantification of phenobarbital, phenytoin, carbamazepine, and its active metabolite carbamazepine 10,11-epoxide

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ARTICLE INFO

Article history:
Received 5 March 2016
Received in revised form
12 May 2016
Accepted 13 May 2016
Available online 17 May 2016

Keywords:
Antiepileptic drugs
Online-SPE-LC-HRMS/MS
Therapeutic drug monitoring

ABSTRACT

A simple and efficient bioanalytical method for simultaneous determination of phenobarbital (PB), phenytoin (PHT), carbamazepine (CBZ), and its active metabolite carbamazepine 10,11-epoxide (CBZE) in human plasma using online solid phase extraction (SPE)-liquid chromatography (LC) coupled with high resolution mass spectrum (HRMS) under targeted MS/MS (t-MS^2) analysis mode has been developed. The procedure integrated an automated sample clean-up of human plasma by Oasis® HLB SPE cartridge, a separation by ZORBAX SB-C18 analysis column, and a quantification by Q-Exactive Hybrid Quadrupole-Orbitrap. The total running time was 13 min. The lower limit of quantification (LLOQ) of PB, PHT, CBZ, and CBZE were 0.008, 0.008, 0.0016 and 0.0016 μg mL^{-1} respectively and the linearities were in the range of 0.008–2.500, 0.008–2.500, 0.0016–0.500 and 0.0016–0.500 μg mL^{-1} respectively. The mean recovery was between 91.82% and 108.27% and the matrix effect was between 93.29% and 102.09%. The relative standard deviations of interday and intraday were less than 6.41%. The method has been successfully applied in therapeutic drug monitoring (TDM) of four Chinese epilepsy patients. This fully automated, simple, sensitive and reliable online-SPE-LC-HRMS/MS method serves well for TDM of PB, PHT, CBZ and CBZE at clinics for either single or combination treatment.

1. Introduction

Therapeutic drug monitoring (TDM) has been widely applied at clinics and well accepted as a means to improve the effectiveness and safety of the antiepileptic drugs (AEDs) such as phenobarbital (PB), phenytoin (PHT) and carbamazepine (CBZ) (Fig. 1). Patients with epilepsy may receive a multiple AEDs regimen for a long to control the disease, even throughout their lives. TDM could be a powerful tool to identify an individual's optimum concentration and to validate bioequivalence in patients [1]. Furthermore, the management of drug interactions and safety related issues is crucial, especially for drugs with narrow treatment indexes, narrow safety window and significant metabolic differences among individuals [2]. Drug interactions are highly dynamic when drugs are added or removed from a multiple drug regimen [3]. When an interacting drug is discontinued from a patients' drug regimen, TDM is conducive for physicians to readjust the dosage of the remaining drugs [4].

PB, PHT and CBZ were developed in 1911, 1938 and 1965 respectively [5–7]. Despite the development of successive generations of AEDs, as the first generation of AEDs, they retain a unique position in the therapeutic arsenal and are still in widespread usage. In routine clinical practice, TDM of PB, PHT, CBZ is applied frequently because of their narrow therapeutic range (15–40 μg mL^{-1}, 10–20 μg mL^{-1} and 4–12 μg mL^{-1}) and their complex pharmacokinetic properties [8]. CBZ was oxidized by inducible hepatic cytochrome P450-dependent enzymes to afford carbamazepine 10,11-epoxide (CBZE) which exerts pharmacological activity as does its parent compound CBZ (Fig. 1) [9]. Toxic symptoms may occur when plasma concentration of CBZE is greater than 3.2 μg mL^{-1}. Thus CBZE quantification is also of great importance in cases where CBZ has been ingested.

In the past decade, there are many technologies for quantification the AEDs in human plasma, including immunological

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http://dx.doi.org/10.1016/j.talanta.2016.05.036
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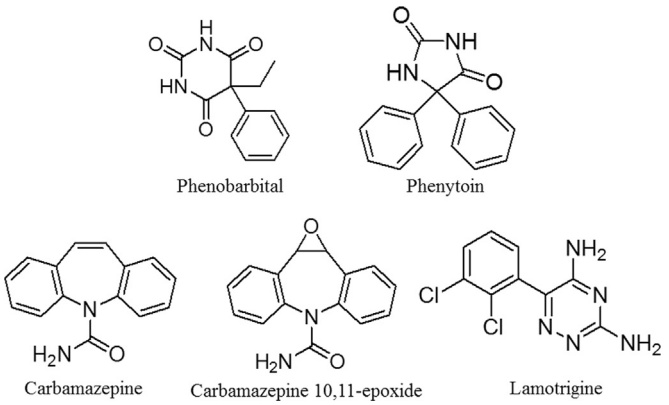


Fig. 1. Chemical structures of the analytes and internal standard.

techniques and chromatographic separation techniques. The immunological techniques consist of fluorescent polarization immunoassay (FPIA) [10–12] and the enzyme-multiplied immunoassay technique (EMIT). While it is easy to perform, an inherent drawback of using immunological techniques is its limit in one drug per testing. Chromatographic separation techniques mainly include high-performance liquid chromatography (HPLC) [8,9,13–19] and LC-MS/MS [20,21] coupled with various sample clean-up methods. However, drug metabolites often share the core structure with their parent drugs after biotransformation and possess near isobaric or isobaric molecular weight [22]. While the methods of HPLC were not able to obtain structure information, the low resolution of MS/MS triple quadrupole (QqQ) may produce false positive results because it lacks the capacity to distinguish compounds with the decimal difference. It is urged to provide accurate mass data to identify the compounds with similar or even identical molecular weight in the biological matrix by high resolving power (RP) of high resolution mass spectrometer (HRMS). As a cutting-edge HRMS platform-the hybrid quadrupole-Orbitrap, well established in proteomic [23], drug metabolism study [24], pesticide screening [25], has been applied in TDM [26]. Hybrid quadrupole-Orbitrap based instruments offer fast positive/negative switching at high scan rates for the simultaneous detection [27], high energy collisional dissociation (HCD) with fragmentation function [28], high selectivity quadrupole for parent ions, resulting in better overall performance.

The sample pretreatment method of above mentioned quantification anti-epileptic techniques includes traditional liquid-liquid extraction (LLE) [16,18], protein precipitation (PP) [8,9,15,21], offline-solid-phase extraction (SPE) [13,20], combined PP/offline-SPE [14], LLE/stir bar-sorptive extraction (SBSE) [17] and PP/micro-extraction by packed sorbent (MEPS) [19], etc. All these methods are time-consuming and labor-intensive. With the advent of the column-switching technique, online-SPE as an efficient, fully automatic sample preparation has been successfully applied in the bioanalysis [29–32]. It increases not only the efficiency, but also the repeatability. Among studies in human fluids (plasma, urine, lymphatic fluid and cerebrospinal fluid, etc), plasma concentration is the most important parameter in clinic management of epileptic patients receiving PB, PHT and CBZ [33]. In our previous work, we have successfully used online-SPE-LC-HRMS to analyze single component in human plasma [34]. In this paper, we developed a sensitive and automated method for simultaneous quantification of multi-components including PB, PHT, CBZ, and its one of active metabolites CBZE in human plasma. The method was validated in terms of linearity, repeatability, accuracy, precision, extraction recovery, matrix effect, stability study and dilution integrity following the Food and Drug Administration (FDA) guidelines [35]. Moreover, the method has been applied at clinics in TDM of four Chinese epilepsy patients.

2. Materials and methods

2.1. Chemicals and reagents

PB (phenobarbital sodium, purity 99.0%) was purchased from Beijing Chemist Science and Technology Co., Ltd (Beijing, China). Both PHT (phenytoin sodium, purity 99.0%) and CBZ (purity 99.0%) were purchased from Shanghai Oriental Pharmaceutical Science and Technology Co. Ltd (Shanghai, China). CBZE (purity 98%) was purchased from Nanjing Chemlin Chemical Industrial Co., Ltd. (Jiangsu, China). Lamotrigine (LTG, purity 99.0%) was used as the internal standard (IS) and purchased from Shanghai Oriental Pharmaceutical Science and Technology Co., Ltd (Shanghai, China).

Ultrapure (18.2 MΩ·cm) water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetonitrile and methanol were of HPLC grade purity purchased from Fisher Scientific (Whitby, ON, Canada). Ammonium acetate, acetic acid and formic acid from Sigma-Aldrich (St. Louis, MO, USA) were used.

Blank human plasma samples from healthy volunteers were kindly donated by Xuanwu Hospital, Capital Medical University. Patients' samples were collected in Xuanwu Hospital, Capital Medical University. All the blank and patients' plasma samples were collected under the approval of the ethical committee of Xuanwu Hospital, Capital Medical University and informed consents were obtained from the volunteers and patients. All blank and patients' plasma samples were collected in K_2EDTA-treated tubes and stored at −80 °C.

2.2. Stock and working solutions

The solutions were prepared and stored according to previous protocol [34]. The standard stock solutions of PB, PHT, CBZ, CBZE and LTG were 4 mg mL^{-1} respectively and stored in brown bottles. Then different volumes of each stock solution were transferred, combined and diluted appropriately with methanol:water (50:50, v/v) for series of concentration. All the stock solutions were stored at −20 °C and the work solutions were stored at 4 °C.

The calibration samples were prepared by spiking blank human plasma with respective working solutions (19:1, v/v). Calibration standards were made at the concentrations of 0.008, 0.020, 0.040, 0.100, 0.200, 0.500, 1.000, 2.500 μg mL^{-1} for PB and PHT, 0.0016, 0.004, 0.008, 0.020, 0.040, 0.100, 0.200, 0.500 μg mL^{-1} for CBZ and CBZE. Quality control (QC) samples were prepared at 0.008 μg mL^{-1} (low concentration of quality control, LQC), 0.200 μg mL^{-1} (middle concentration of quality control, MQC), 2.500 μg mL^{-1} (high concentration of quality control, HQC) for PB and PHT and 0.0016 μg mL^{-1} (LQC), 0.040 μg mL^{-1} (MQC), 0.500 μg mL^{-1} (HQC) for CBZ and CBZE. All samples were stored at 4 °C before analysis.

10 μL of Internal standard working solution (0.500 μg mL^{-1} LTG) was added to 200 μL volume of calibration plasma sample. Then the sample was vortexed for 30 s After centrifugation (5 min, 13,000 rpm), the supernatant was transferred into auto sampler vial and 10 μL sample was injected to online-SPE-LC-HRMS/MS system.

2.3. Online-SPE and LC parameters

Samples were pretreated and separated using a Thermo Scientific Dionex Ultimate 3000 system (Thermo Scientific Fisher, Waltham, MA and Dionex Softron GMbH Part of Thermo Fisher Scientific, Germany). The SPE cartridge was a Waters Oasis®HLB

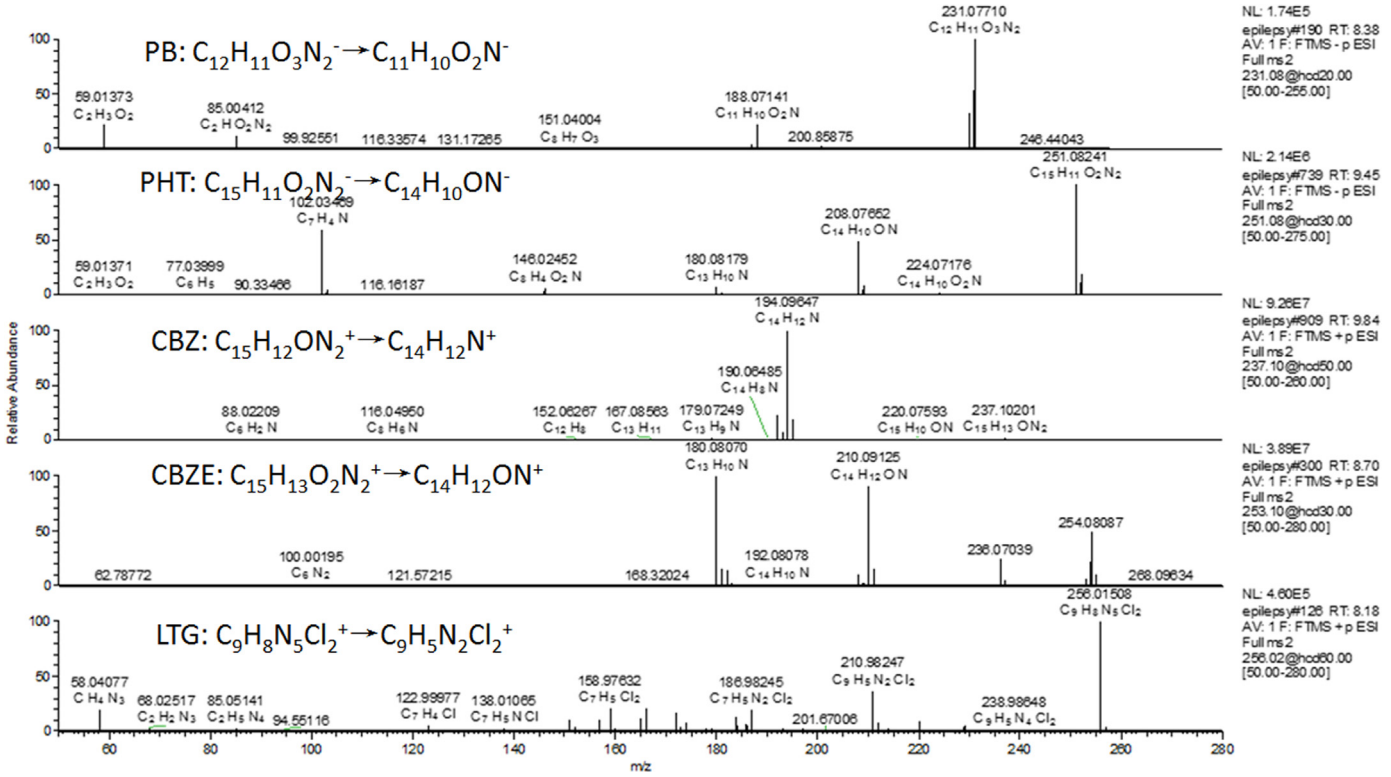


Fig. 2. The MS^2 spectrum of analysts in t-MS^2 mode.

cartridge with a dimension of 2.1 mm × 20 mm id and 5 μm particle size. The reversed-phase analytical column was a Agilent ZORBAX SB-C18 column with a dimension of 4.6 mm × 250 mm id and 5 μm particle size.

The flow scheme for automated online-SPE-LC-HRMS/MS has been described in the previous paper [34]. A standard process consists of three steps (loading, transfer and separation). In the loading step, 10 μL of plasma sample was directly injected into the SPE cartridge by autosampler with the six-port injector valve in 6–1 position, and the matrix interferences were washed out by acetonitrile-10 mM ammonium acetate buffer 2:98 (v/v, pH=3.5) at the flow rate of 1 mL min^{-1} for 2 min, while the target analytes (PB, PHT, CBZ, CBZE and LTG) were reserved on the SPE cartridge. Meanwhile, a second pump conditioned the analytical column with an initial 10:20:70 ($v/v/v$) eluent of acetonitrile-methanol-10 mM ammonium acetate buffer (pH=5.5) at the flow rate of 1 mL min^{-1}. In the transfer step, the six-port valve was switched to 2-1 position, SPE cartridge was coupled with the analytical column. Then target analytes were transferred from SPE cartridge to the analytical column by gradient elution. In the separation step, the six-port valve was switched back to 6-1 position, the SPE cartridge and analytical column were at parallel stage once again. The best possible chromatographic separation of PB, PHT, CBZ, CBZE and LTG was achieved according to Table 1. In order to avoid the clogging of columns and prolong the column life time, the SPE cartridge and analysis column were washed every 3 injections.

2.4. Electrospray ionization source

The HRMS system was equipped with a heated electrospray ionization source (HESI-II) and Orbitrap mass analyzer. The MS system for PB and PHT was operated in negative mode, the MS system for CBZ, CBZE and LTG was operated in positive mode. Source parameters were as follows: the spray voltage of positive and negative was 3.5 kV and 4 kV, respectively; the S-lens RF level was 50 V; the aux gas heater temperature and capillary temperature were adjusted to 300 °C and 320 °C, respectively. Ultrapure liquid nitrogen was used as ion source gas and collision gas, the sheath gas flow rate and aux gas flow rate were set to 35 and 10 (arbitrary units), respectively.

Table 1
Online-SPE and HPLC conditions.[a]

Time (min)	SPE cartridge			Analytical column			Valve switching
	Flow rate (mL min^{-1})	A (%)	B (%)	Flow rate (mL min^{-1})	A (%)	C (%)	D (%)	
0	1	2	98	1	10	20	70	6-1
2	1	2	98	1	10	20	70	2-1
3	1	2	98	1	10	30	60	6-1
6	1	100	0	1	10	60	30	
11	1	100	0	1	60	10	30	
11.5	1	2	98	1	10	20	70	
13	1	2	98	1	10	20	70	

[a] A, acetonitrile B, 10 mM ammonium acetate buffer (pH=3.5, adjusted by acetic acid) C, methanol D, 10 mM ammonium acetate buffer (pH=5.5, adjusted by acetic acid).

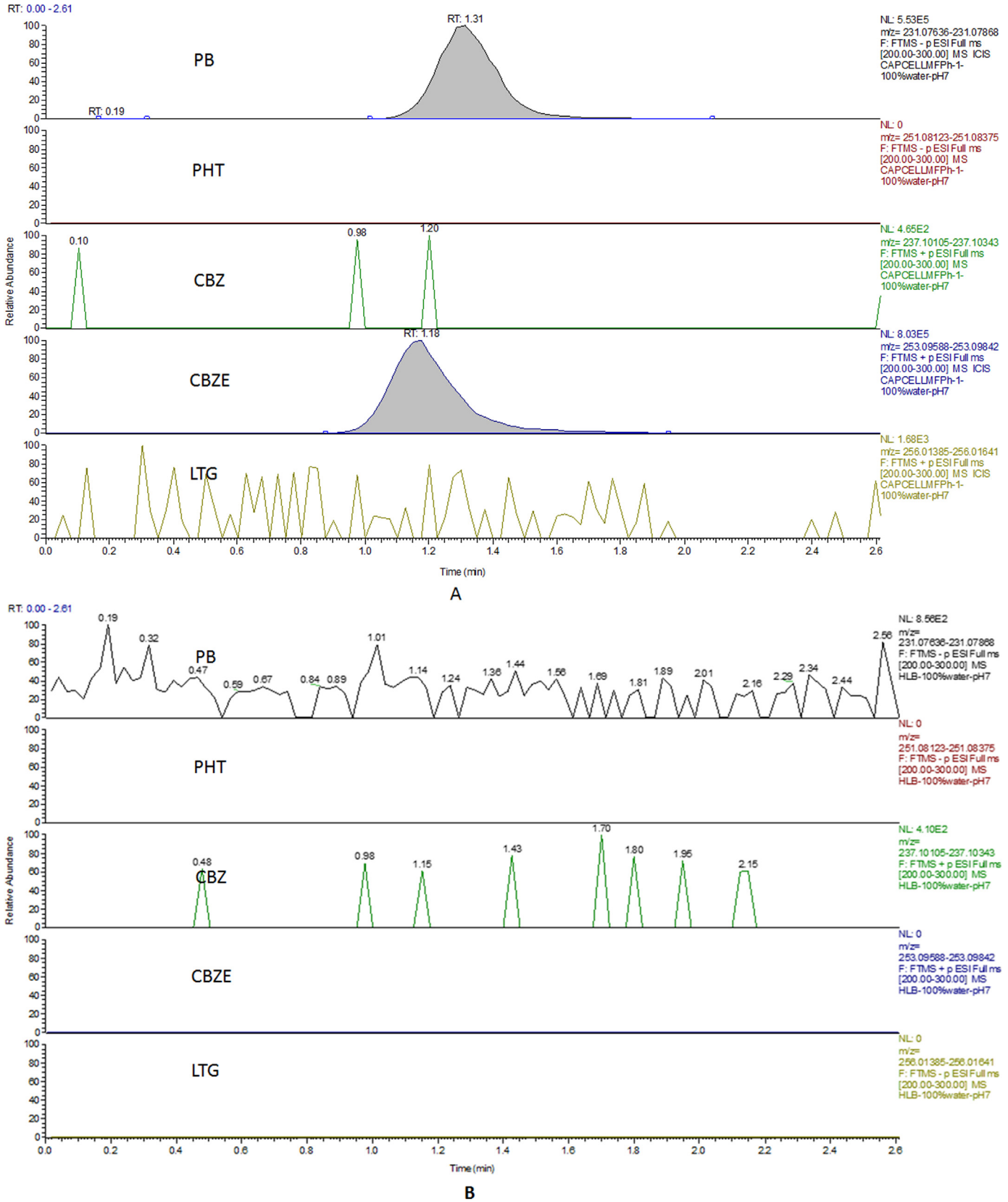


Fig. 3. The retention effects of CAPCELL MF pH-1 cartridge (A) and Waters® HLB cartridge (B).

2.5. *Quantification method parameters*

All quantitative data in this study were acquired using t-MS^2 scan mode. The mass resolution was set at 17,500 FWHM (m/z 200), automatic gain control (AGC) target was set at 2×10^5 with a maximum injection time (IT) of 100 ms. As long as the targeted compounds were detected in a mass inclusion list, precursor ions selected by the quadrupole were sent to the HCD collision cell of

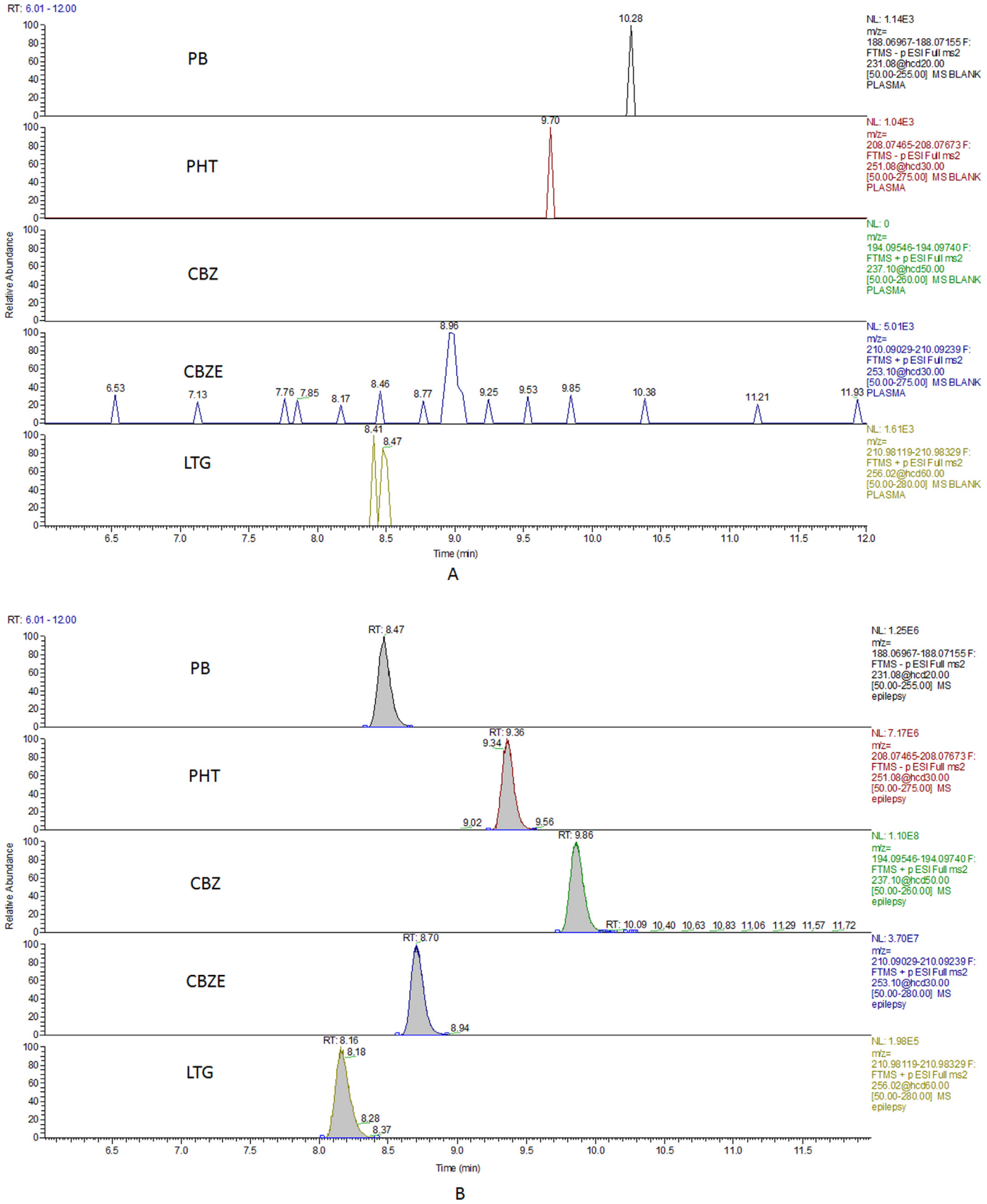
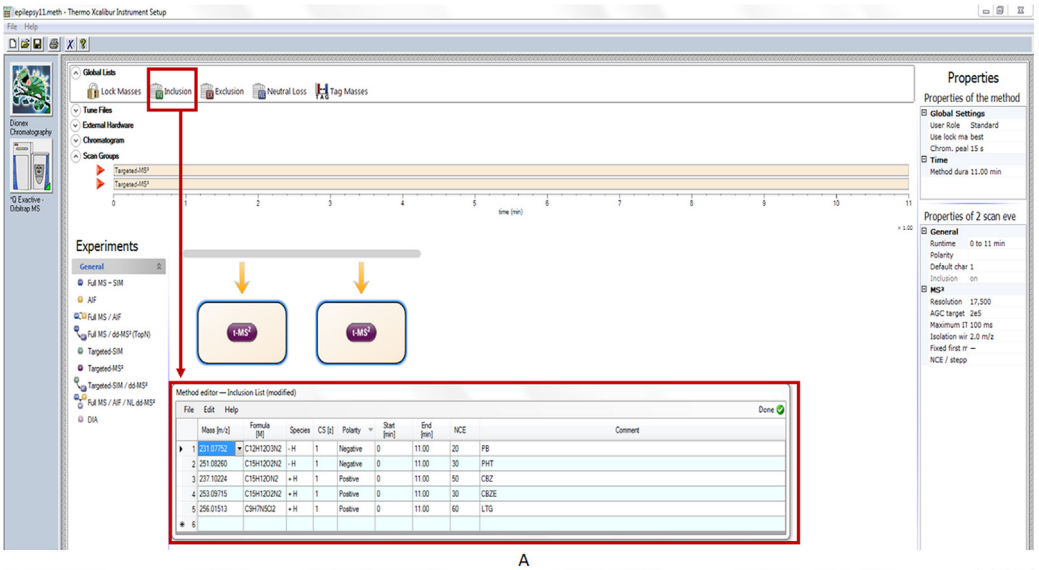
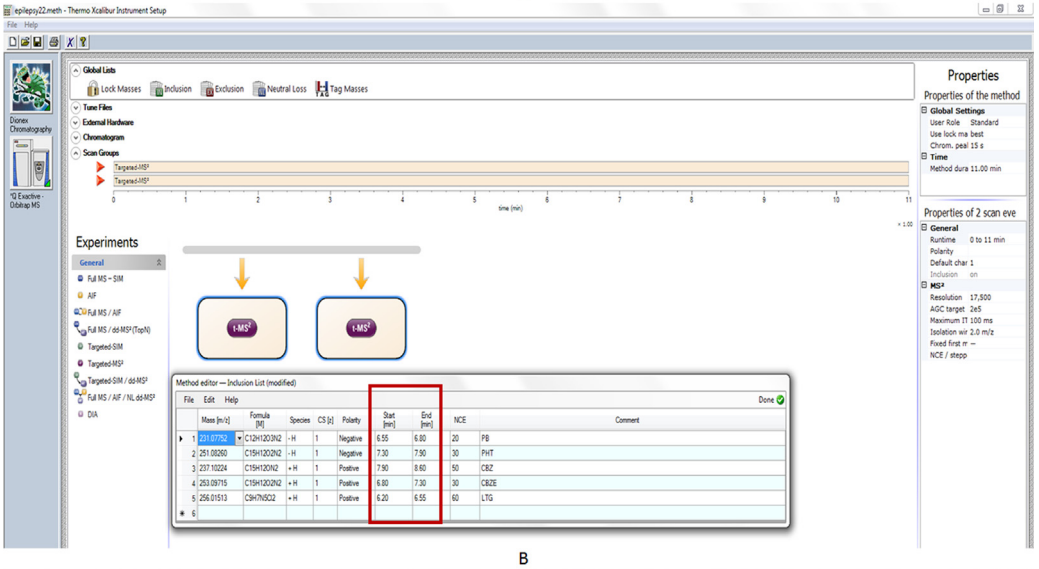


Fig. 4. The chromatograms of blank plasma (A) and plasma spiked with PB, PHT CBZ, CBZE, LTG (B).

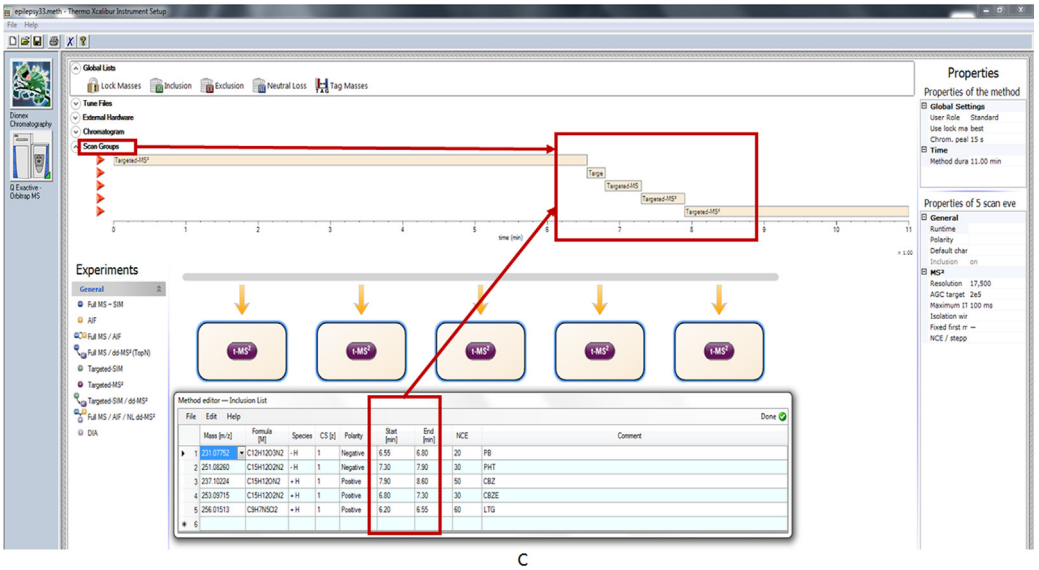
the Q-Orbitrap mass spectrometer. The precursors were fragmented with specific collision energy (NCE) to obtain product ions spectra (Fig. 2). Using this strategy, high response values of the quantification of known analytes was facilitated in a single run analysis.



A



B



C

Fig. 5. t-MS^2 setting in negative and positive mode.

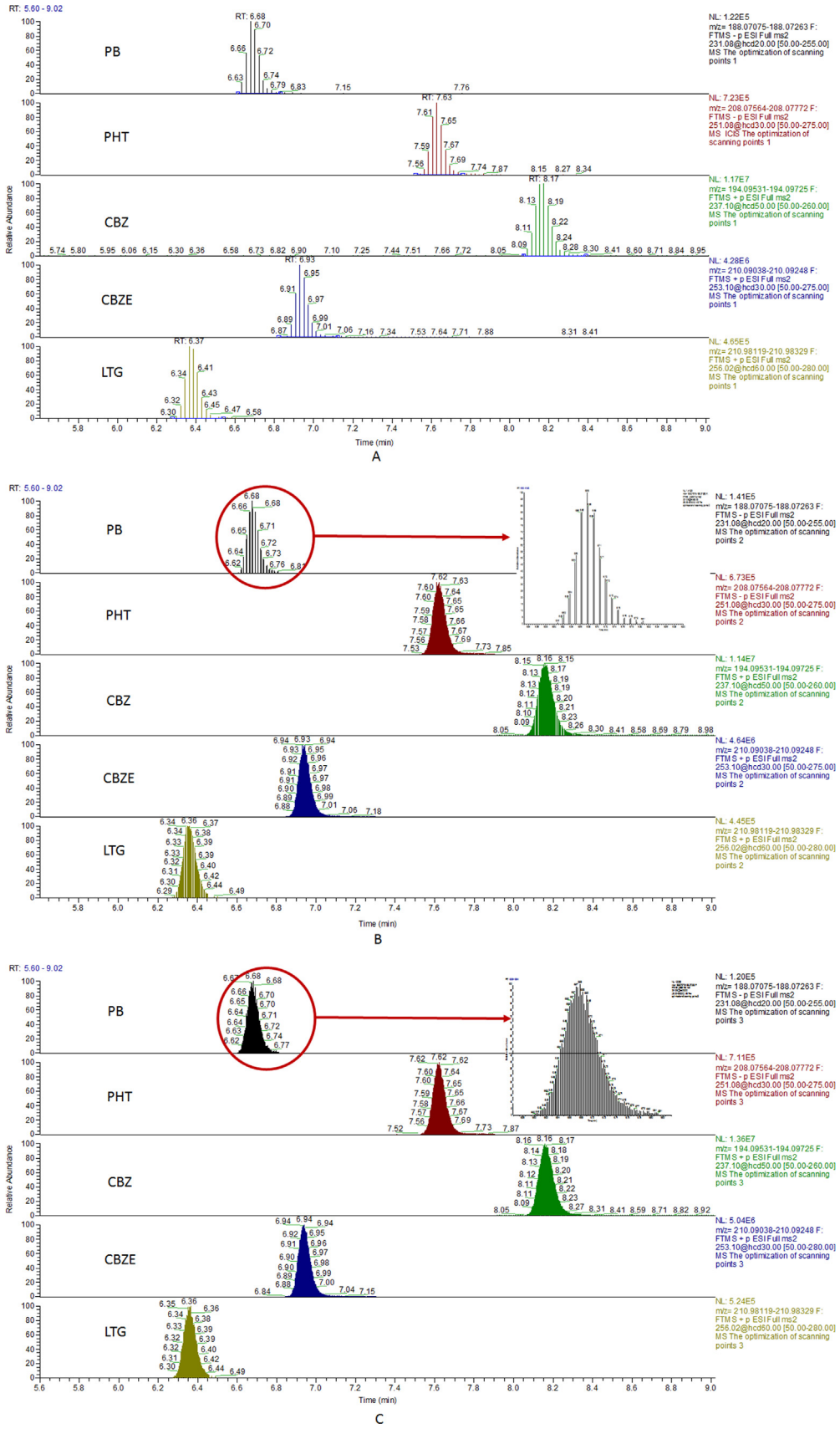


Fig. 6. Extracted ion chromatographic peak (stick display) of PB, PHT CBZ, CBZE and LTG in different t-MS^2 setting.

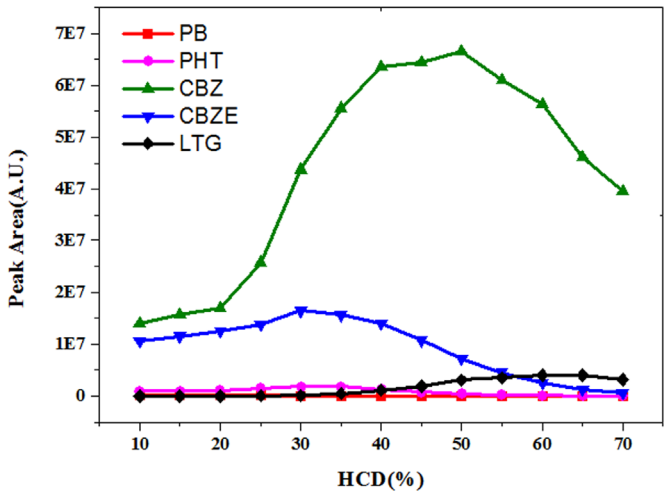


Fig. 7. t-MS^2 fragmentation mass spectra of target compounds acquired with respective NCE. (Major products ions used for quantitation in bold).

2.6. Data analysis and method validation

Detection of PB, PHT, CBZ, CBZE and LTG was performed using a Q-Exactive mass spectrometer controlled by the Xcalibur 3.0 software (Thermo Fisher Scientific, Waltham, MA). The molecular formulas and calculated exact mass of the parent and product ions were obtained using the Mass Frontier 7.0 software (Highchem, Bratislava, Slovakia). Both positive and negative identification of analytes were based on the accurate mass of the analytes with less than ±5 ppm error, retention time comparison within ±0.03 min.

2.6.1. Accuracy and precision

Repeatability was evaluated at QC samples five replicates on a single workday (intra-day precision) and prepared daily on a period of 3 days (inter-day precision). Accuracy values were determined by the relative error (%) and precision values were defined as the relative standard deviation (RSD%).

2.6.2. Extraction recovery and matrix effect

Recoveries were determined as a ratio of the response of the analytes recorded for spiked human plasma by online-SPE-LC-HRMS/MS to the response of the analytes obtained for a blank matrix solution by LC-HRMS/MS expressed as a percentage at three QC samples. Matrix effects were determined as a ratio of the response of the analytes recorded for a blank matrix solution by LC-HRMS/MS to the response of the analytes obtained for an initial mobile phase solution by LC-HRMS/MS expressed as a percentage at three QC samples. The blank matrix solution was obtained following the reported technique [36]: 10 μL human blank plasma was loaded and transferred with the method described above, the acetonitrile in the transfer extraction solution was swept out with nitrogen, and then stored in 4 °C. In LC-HRMS/MS process, the condition of mobile phase was achieved according to the transfer and separation condition of Table 1.

Table 3
The accuracy of HQC and R^2 under different concentration range.

Analytes	Linear range of quantification (μg mL^{-1})	R^2	The accuracy of HQC
PB	0.008–2.500	0.9990	96.76
	0.008–5.000	0.9977	92.58
PHT	0.008–2.500	0.9974	104
	0.008–5.000	0.9978	99.72
CBZ	0.0016–0.500	0.9969	90.2
	0.0016–1.000	0.9901	80.74
CBZE	0.0016–0.500	0.9971	90.1
	0.0016–1.000	0.9898	79.56

2.6.3. Stability study

Stability testing was evaluated the stability of the analytes at room temperature for 6 h and at −80 °C for 30 days and freeze-thaw stability study at −80 °C for 3 cycles.

2.6.4. Clinical application

The validated method was applied for the quantification of PB, PHT, CBZ and CBZE in plasma samples of epileptic patients treated with PB, PHT or CBZ at Xuanwu Hospital, Capital Medical University. The blood samples were taken in the morning before the daily administration of the AEDs, complying with the protocol approved by Xuanwu Hospital, Capital Medical University and the informed consent was obtained from each subject.

3. Results and discussion

3.1. Optimization of SPE and LC parameters

One of the most promising strategies to reduce manual sample preparation to a minimum, remove the greatest amount of matrix interferents and achieve the maximum sensitivity for anti-epileptic drugs in LC-HRMS/MS methods is the use of online-SPE. An appropriate SPE cartridge is crucial to achieve a complete extraction of the analytes, the maximum removal of matrix in the loading sample and a rapid transfer from the SPE cartridge to the separation column [37]. Two cartridges, Oasis®HLB cartridge (2.1*20 mm, 5 μm) and CAPCELL MF pH-1 cartridge (4.0*10 mm, 5 μm), were evaluated by loading a 10 μL standard sample

Table 2
A summary of reported quantification method and linear range of PB, PHT, CBZ and CBZE in human plasma.

Ref.	pretreatment	Analysis method	PB (μg mL^{-1})	PHT (μg mL^{-1})	CBZ (μg mL^{-1})	CBZE (μg mL^{-1})
[8]	PP	HPLC-UV	5–100	1–50	1–25	1–10
[9]	Offline-SPE	HPLC-UV	–	–	0.05–30	0.1–30
[13]	Offline-SPE	HPLC-UV	–	–	0.3–60	0.3–50
[14]	PP/offline-SPE	HPLC-UV	0.25–100	0.5–50	0.1–50	0.1–50
[15]	PP	HPLC-UV	0.9–38.7	2.2–48.5	4.1–42.3	0.5–21.2
[16]	LLE	HPLC-UV	1.5–60	1.25–50	–	–
[17]	LLE/SBSE	HPLC-UV	0.125–40	0.125–40	0.08–40	0.08–40
[18]	LLE	HPLC-UV	–	–	2–20	–
[19]	PP/ MEPS	HPLC-UV	0.2–40	0.3–30	0.1–15	0.1–5
[20]	Offline-SPE	HPLC-MS	0.5–40	0.5–40	0.3125–25	0.3125–25
[21]	PP	UPLC-MS/MS	5–50	2.2–53.8	1.3–13.5	0.9–23.8

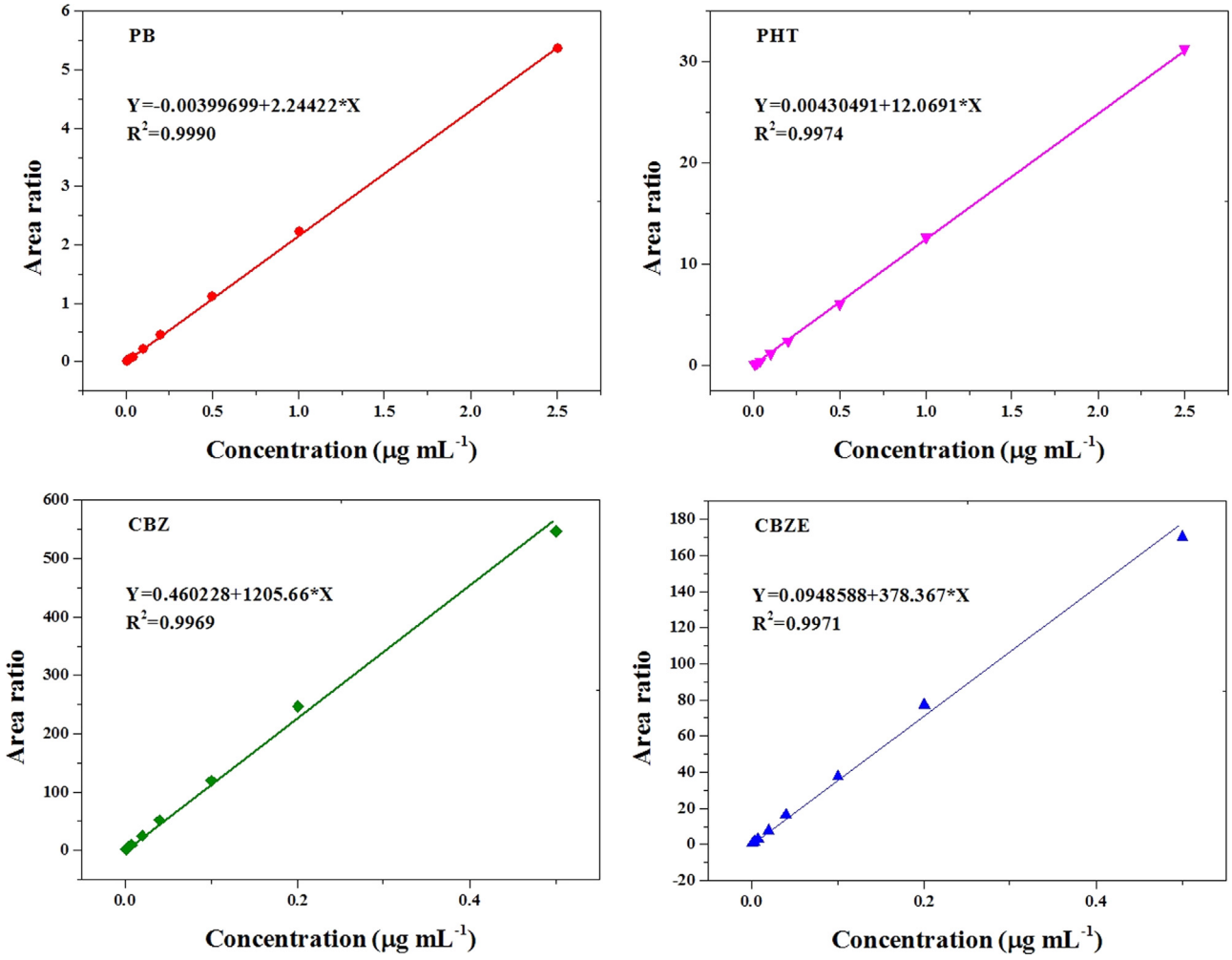


Fig. 8. Data of linear regression equation of PB, PHT, CBZ and CBZE.

Table 4
Intraday and interday precision and accuracy.

Analyte	Nominal concentration (μg mL^{-1})	Intra-batch				Inter-batch			
		N	Mean concentration found (μg mL^{-1})	Accuracy (%)	RSD (%)	N	Mean concentration found (μg mL^{-1})	Accuracy (%)	RSD (%)
PB	0.008	5	0.00810	101.28	5.11	15	0.00873	109.13	6.41
	0.200	5	0.19595	97.98	1.80	15	0.19839	99.19	4.97
	2.500	5	2.48211	99.28	0.79	15	2.47923	99.17	2.50
PHT	0.008	5	0.00788	98.5	3.98	15	0.00763	95.42	3.91
	0.200	5	0.19281	96.40	0.71	15	0.19698	98.49	4.76
	2.500	5	2.67715	107.09	1.56	15	2.66967	106.79	2.01
CBZ	0.0016	5	0.00164	102.78	1.02	15	0.00156	97.66	6.19
	0.040	5	0.04286	107.17	0.62	15	0.04080	102.01	3.80
	0.500	5	458.186	89.63	1.36	15	0.45142	90.28	2.05
CBZE	0.0016	5	0.00158	98.46	2.05	15	0.0155	97.02	4.22
	0.040	5	0.043.38	108.45	1.07	15	0.04248	106.21	2.45
	0.500	5	0.46718	89.46	1.72	15	0.44112	88.22	1.73

(0.200 μg mL^{-1}) on SPE-HRMS/MS with ultrapure water at a flow rate of 1 mL min^{-1}. Oasis®HLB cartridge showed good performance for all analytes, while CAPCELL MF pH-1 exhibited poor retentive efficiency for PB and CBZ in two min (Fig. 3). This may be explained by the properties of the polyoxyethylene-phenyl phase of the CAPCELL MF pH-1 cartridge allowing non-polar and π–π interactions. In contrast, Oasis®HLB column consists of a mixed stationary phase enabling both hydrophilic and lipophilic interactions. Therefore it may better retain the moderate polar chemicals. The next step is the transfer from SPE cartridge to analytical column. Transfer was completed in 1 min while the mobile phase was 20% ACN. Oasis®HLB gave good elution efficiency and was selected for the method development. The target analytes were fully separated by C18 column at 40 °C under gradient elution and each compound's peak was symmetrical (Fig. 4). Retention times for analyst were: LTG (8.05 min), PB (8.45 min), CBZE (8.69 min), PHT (9.35 min) and CBZ (9.85 min).

Table 5
Matrix effect and extraction recovery.

Analyte	Nominal concentration ($\mu g\ mL^{-1}$)	Extraction recovery (%)		Matrix effect (%)	
		Mean	RSD (%)	Mean	RSD (%)
PB	0.008	103.12	3.66	102.09	4.81
	0.200	108.27	3.51	100.15	3.09
	2.500	98.35	1.9	98.93	1.86
PHT	0.008	102.42	2.94	98.99	4.30
	0.200	103.09	2.53	95.17	3.05
	2.500	95.39	1.19	98.11	1.65
CBZ	0.0016	102.74	2.54	96.29	2.98
	0.040	100.82	1.98	93.29	2.20
	0.500	91.82	1.73	96.71	1.52
CBZE	0.0016	95.28	4.77	94.52	4.78
	0.040	92.17	1.31	96.19	2.08
	0.500	93.27	2.48	97.66	1.09

Table 6
Stability study under different conditions.

Analyte	Stability	Storage conditions	Level[a] ($\mu g\ mL^{-1}$)	Mean comparison samples[b] ($\mu g\ mL^{-1}$)	Mean stability samples[c] ($\mu g\ mL^{-1}$)
PB	Short-term	Room temperature for 6 h	0.008	0.00810	0.00836
			0.200	0.19595	0.19357
			2.500	2.48211	2.48688
	Freeze-thaw	At −80 °C for 3 cycles	0.008	0.00911	0.00917
			0.200	0.18860	0.18859
			2.500	2.43521	2.41457
	Long-term	At −80 °C for 30 days	0.008	0.00898	0.00853
			0.200	0.21062	0.20258
			2.500	2.54101	2.53892
PHT	Short-term	Room temperature for 6 h	0.008	0.00788	0.00826
			0.200	0.19281	0.18922
			2.500	2.67715	2.65639
	Freeze-thaw	At −80 °C for 3 cycles	0.008	0.00745	0.00757
			0.200	0.19039	0.18827
			2.500	2.63748	2.64060
	Long-term	At −80 °C for 30 days	0.008	0.00748	0.00762
			0.200	0.21841	0.21389
			2.500	2.71206	2.70180
CBZ	Short-term	Room temperature for 6 h	0.0016	0.00164	0.00165
			0.040	0.04286	0.04145
			0.500	0.44819	0.44852
	Freeze-thaw	At −80 °C for 3 cycles	0.0016	0.00144	0.00150
			0.040	0.03957	0.03976
			0.500	0.46055	0.46045
	Long-term	At −80 °C for 30 days	0.0016	0.00160	0.00155
			0.040	0.03997	0.03827
			0.500	0.46068	0.45909
CBZE	Short-term	Room temperature for 6 h	0.0016	0.00158	0.00159
			0.040	0.04338	0.04282
			0.500	0.44718	0.43948
	Freeze-thaw	At −80 °C for 3 cycles	0.0016	0.00148	0.00141
			0.040	0.04124	0.04109
			0.500	0.46029	0.45671
	Long-term	At −80 °C for 30 days	0.0016	0.00151	0.00167
			0.040	0.04027	0.04007
			0.500	0.46077	0.45824

[a] Level was referred to as the concentrations of quality control samples (LQC, MQC and HQC).
[b] Mean comparison samples were referred to as the mean sample concentrations measured immediately after calibration samples were prepared. The number of the testing was greater than five.
[c] Mean stability samples were referred to as the mean sample concentrations measured after calibration samples were stored. The number of the testing was greater than five.

Table 7
Dilution integrity study of analytes.

Analyte	N[a]	Dilution integrity[b]	Nominal Concentration ($\mu g\ mL^{-1}$)	Mean concentration found ($\mu g\ mL^{-1}$)	Accuracy	RSD (%)
PB	5	2	5.000	4.771	95.42	2.31
	5	5	5.000	4.762	95.24	1.72
	5	25	50.000	51.575	103.15	3.02
PHT	5	2	5.000	5.070	101.40	2.18
	5	5	5.000	4.773	95.46	2.56
	5	25	50.000	53.300	106.60	1.38
CBZ	5	2	1.000	0.877	87.70	3.22
	5	5	1.000	0.923	92.30	1.29
	5	25	10.000	9.737	97.37	2.98
CBZE	5	2	1.000	0.885	88.50	3.41
	5	5	1.000	0.905	90.50	2.83
	5	25	10.000	10.032	100.32	1.49

[a] N was referred to as the number of determinations per concentration.
[b] Dilution integrity was referred to the ratio of the dilution.

3.2. HRMS/MS parameters optimization

3.2.1. Selection of product ions

Due to the different responses of compounds in different detection mode (positive ion and negative ion), it is important to select an appropriate mode. In the present study, PB and PHT were negative ions, while CBZ and CBZE were positive ions. The MS/MS fragmentation of PB, PHT, CBZ and CBZE yielded suitably abundant and characteristic fragment ions to be used as quantification ions, m/z 231.07752 → m/z 188.07061, m/z 251.08249 → m/z 208.07569, m/z 237.10224 → m/z 194.09643 and m/z 253.09715 → m/z 210.09134.

3.2.2. Optimization of scanning points

The t-MS^2 acquired MS^2 scans based on the entries of the inclusion list. The precursor ions in the inclusion list were selected by quadrupole, then fragmented in HCD cell, followed by collection in the C-trap, finally analyzed in the orbitrap. The number of data scan points per chromatographic peak largely depended on the setting in the inclusion list. When the same time window was used for all target analytes (three positive ions and two negative ions) (Fig 5(A)), 8–10 data points were obtained and the average screening interval is 1200–1300 ms (Fig. 6(A)). When the data acquisition time was limited (Fig. 5(B)), about 50 data points were acquired for each analyte except 28 data points for PB (Fig. 6(B)). This is because RT of PB (negative) was in between LTG (positive) and CBZE (positive). After RT and suitable scanning mode of each ion were studied, scan groups were optimized (Fig 5C). Under this condition, a single mode was performed within the corresponding RT of a particular ion. To our satisfaction, eventually the scanning points for all the target analytes were more than 70 points (Fig. 6 (C)). In the meantime, the scanning speed was improved significantly.

3.2.3. Optimization of NCE

Fragmentation energy scans were carried out to obtain the optimal NCE for high response of product ions. The tMS^2 parameters were optimized using individual standards at a concentration of 200 $\mu g\ L^{-1}$. 10 μL Sample was injected to ZORBAX SB-C18 column by autosampler at a flow rate of 1 $mL\ min^{-1}$, one fifth of the sample was split into MS. The extracted peak areas of product ions changed with the increase of NCE using the Q-Exactive Tune 3.0 software (Fig. 7). The optimization results for PB, PHT, CBZ, CBZE and LTG were 20%, 30%, 50%, 30% and 60%, respectively.

Table 8
Plasma concentrations of PB, PHT, CBZ and CBZE in real plasma samples obtained from epileptic patients under treatment with different therapeutic regimens.

Number	sex	age	Dosage	Times	Concentration ($\mu g\ mL^{-1}$)
1	F	26	0.4 g CBZ sustained-release tablet and 0.05 g Topiramate tablet	bid[a]	CBZ 9.01, CBZE 1.38
2	F	29	0.4 g CBZ sustained-release tablet	bid	CBZ 10.8, CBZE 0.72
3	M	28	0.2 g CBZ sustained-release tablet and 0.5 g VPA sustained-release tablet	tid[b]	CBZ 4.38, CBZE 0.52
4	M	27	0.1 g CBZ tablet, 0.2 g VPA, 0.03 g PB tablet and 0.1 g PHT tablet	bid	PHT0.23, PB 13.3, CBZ 1.66, CBZE 0.26

[a] Bid was referred to as giving medication twice a day.
[b] Tid was referred to as giving medication three times a day.

3.3. Method validation

3.3.1. Selectivity

The chromatography of blank plasma and spiked plasma sample with PB, CBZ, PHT and CBZE at LLOQ were shown in Fig. 4. Interference from endogenous materials was not observed at the retention time of the target analytes and the internal standard indicating that the method was selective.

3.3.2. Linearity and LLOQ

The reported linearity ranges of PB, PHT, CBZ and CBZE in human plasma were summarized in Table 2. In the current study, the R^2 value and accuracy of HQC under various concentrations were examined. As shown in Table 3, the HQC of the CBZ and CBZE was set as $0.5\ \mu g\ mL^{-1}$. Considering the dilution integrity of the complex, the concentration of $2.500\ \mu g\ mL^{-1}$ was selected as the HQC of PB and PHT. Linearity was assessed with plasma samples spiked with known concentrations of PB and PHT over a concentration range of 0.008–$2.500\ \mu g\ mL^{-1}$, CBZ and CBZE over a concentration range of 0.0016–$0.500\ \mu g\ mL^{-1}$ ($R^2 > 0.99$). The LLOQs were $0.008\ \mu g\ mL^{-1}$ for PB and PHT, $0.0016\ \mu g\ mL^{-1}$ for CBZ and CBZE. The linear relationship between the chromatographic peak area ratio and the different concentration analytes was investigated by Xcalibur 3.0. Data of linear regression equation of the analytes was listed in Fig. 8.

3.3.3. Accuracy and precision

The accuracy and precision of QC samples were shown in Table 4. The accuracy of the analytes was between 88.22% and 108.45% while the RSD% of QC samples was between 0.62% and 6.41%.

3.3.4. Extraction recovery and matrix effect

Results of extraction recovery and matrix effect of the analytes were listed in Table 5. The extraction recovery was from 91.82% to 108.27% with the matrix effect ranging from 93.29% to 102.09%.

3.3.5. Stability study

Data of the stability experiments were listed in Table 6 for each analyte. PB, CBZ, PHT and CBZE in human plasma were stable at room temperature for 6 h and at $-80\ ^{\circ}C$ for 30 days. Freeze-thaw stability study at $-80\ ^{\circ}C$ for 3 cycles was carried out. The result showed that the analytes were stable under the above conditions.

The mean calculated dilution factors of one-fifth and half dilution of $2\times$ the highest concentration, and one-twenty five of $20\times$ the highest concentration were between 87.77% and 106.60% of the nominal values while the value of RSD% ranged from 1.29% to 3.41%. Data of the dilution integrity study were shown in Table 7.

3.3.6. Quantification of PB, CBZE, PHT and CBZ in patients' specimens

The established method has been successfully applied in the TDM of four Chinese patients with epilepsy. The dosage regimen of patient 1 was 0.4 g CBZ sustained-release tablet and 50 mg topamax tablet twice a day, the dosage regimen of patient 2 was 0.4 g CBZ sustained-release tablet twice a day, the dosage regimen of patient 3 was 0.5 g sodium valproate (VPA) sustained-release tablet and 0.2 g CBZ sustained-release tablet three times a day, while the dosage regimen of patient 4 was 0.1 g CBZ tablet, 0.2 g VPA tablet, 0.03 g phenobarbital sodium tablet and 0.1 g phenytoin sodium tablet twice a day. The specimens were collected before ingestion of morning dosing. Results were shown in Table 8. The CBZ concentrations were $9.01\ \mu g\ mL^{-1}$, $10.8\ \mu g\ mL^{-1}$, $4.38\ \mu g\ mL^{-1}$ and $1.66\ \mu g\ mL^{-1}$ for patient 1–4, respectively. The CBZE concentrations were $1.38\ \mu g\ mL^{-1}$, $0.72\ \mu g\ mL^{-1}$, $0.52\ \mu g\ mL^{-1}$ and $0.26\ \mu g\ mL^{-1}$ for patient 1–4, respectively. The concentrations of PB and PHT were $0.23\ \mu g\ mL^{-1}$ and $13.3\ \mu g\ mL^{-1}$, respectively, for patient 4. All the measured drug concentrations of patients samples were generally correlated to the drug dosage and not beyond the therapeutic range. Under the help of our efficient method, the doctor could monitor the concentration immediately and accurately. This method may facilitated the doctor adjust dosage at clinics.

4. Conclusion

An automated online-SPE-LC-HRMS/MS method for the sensitive and accurate determination of PB, PHT, CBZ and CBZE in human plasma has been developed. The online analysis allowed the operating time of 13 min. It offers a number of advantages, such as rapid sample preparation (direct sample injection), small sample analysis volume (10 μL), high sensitivity and selectivity (t-MS^2 mode). Under the optimized condition, the proposed analytical procedure was validated in terms of linearity, repeatability, accuracy and precision following the FDA guidelines. The method has been successfully applied to the TDM for four Chinese epilepsy patients.

Acknowledgements

This work was supported by the National Basic Research Program of China (973 program, Grant Nos. 2013CB911104 and 2013CB911100), the National Natural Science Foundation of China (Grant No. 21572116), the Fundamental Research Funds for the Central Universities (Grant No. 20141081129) and the Tianjin Science and Technology Program (Grant Nos. 13JCYBJC24300 and 13JCQNJC13100).

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