Journal of Chromatography B, 879 (2011) 1939–1944

Contents lists available at ScienceDirect

Journal of Chromatography B

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

LC–MS/MS method for simultaneous determination of valproic acid and major metabolites in human plasma

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ARTICLE INFO

Article history:
Received 14 March 2011
Accepted 13 May 2011
Available online 23 May 2011

Key words:
Liquid chromatography-tandem mass spectroscopy
Valproic acid
Metabolites
Human plasma
Epileptic patients

ABSTRACT

A rapid and sensitive method using liquid chromatography-tandem mass spectroscopy (LC–MS/MS) was developed and validated for simultaneous quantitative determination of valproic acid and three major metabolites (3-OH-valproic acid, 4-ene-valproic acid and 5-OH-valproic acid) in human plasma. The analytes and internal standard were isolated from 200 μL samples by solid phase extraction using a ZORBAX SB-C_8 column (3.5 μm, 2.1 × 100 mm) with an isocratic mobile phase consisting of methanol-10 mM ammonium acetate (80:20, v/v) containing 0.1% formic acid at a flow rate of 0.3 mL/min. The method had a chromatographic total run time of 2.0 min. The lower limit of quantification of valproic acid, 3-OH-valproic acid, 4-ene-valproic acid and 5-OH-valproic acid of the method was 2030, 51.5, 50.15 and 51.25 ng/mL, respectively. The method was linear for valproic acid and the three metabolites with correlation coefficients >0.995 for all analytes. The intra-day and inter-day accuracy and precision of the assay were less than 15.0%. This analytical method was successfully used to assay plasma concentrations of valproic acid and the three metabolites in human plasma from epileptic patients.

1. Introduction

Valproic acid (2-propylpentanoic acid, VPA) is a broad-spectrum antiepileptic drug with unique anticonvulsant properties and used in the treatment of epilepsy and mood disorders. Therapeutic use of VPA has been associated with a rare, but severe and often fatal hepatotoxicity characterized by steatosis with or without necrosis of the liver [1]. Metabolism by CYP pathway is not a prominent route of VPA elimination. VPA is almost completely eliminated by metabolism, with less than 4% of an administered dose excreted unchanged into the urine [2]. The biotransformation of VPA involves three major metabolic ways, including uridine diphosphate glucuronosyltransferases (UDPGT)-mediated pathway, mitochondrial β-oxidation way and cytochrome P-450 (CYP2C9, CYP2C19 and CYP2A6) way, accounting for 50%, 40% and 10% of a dose, respectively [3].

Valproic acid has complex metabolic pathways despite its simple structure (shown in Fig. 1A). Major metabolic pathways of VPA are glucuronidation and beta-oxidation as a fatty acid [4]. The initial metabolite by mitochondrial beta-oxidation is 2-propyl-2-pentenoic acid (2-ene), which is one of the pharmacologically active unsaturated metabolites of VPA. This metabolite is further hydroxylated to 3-hydroxy valproate (3-OH), while it has been shown that 3-ene is reversibly formed by isomerization of 2-ene [5]. Thus, 2-ene, 3-ene, and 3-OH are regarded as beta-oxidation-associated metabolites of VPA. Meanwhile, it has been reported that the metabolism of VPA to 4-ene, 4-OH and 5-OH is catalyzed by microsomal oxidation [6]. Especially, it has been specified that the formation of 4-ene is mediated by CYP2C9 [7,8]. In relation to VPA toxicity, some reports have suggested that some unsaturated metabolites, especially 4-ene, may be at least partly associated with VPA-associated hepatotoxicity [9] and teratogenicity [4].

The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, selectivity, high sensitivity, small volume requirements and rapid turnaround time. Several methods for quantification of VPA in plasma have been described, and most of them have been based on immunological methods [10] and gas chromatographic procedures with the flame ionization detector (FID) [11,12] or mass spectrometric (MS) detection [13–15]. Although desired sensitivity has been achieved using GC–MS/MS, prior derivatization limits its suitability for routine sample analysis. Capillary electrophoretic techniques [16] have also been employed with UV detection to quantify VPA in plasma samples. High-performance liquid chromatography (HPLC) analysis of VPA with direct UV detection [17,18] also has problems related to

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doi:10.1016/j.jchromb.2011.05.022

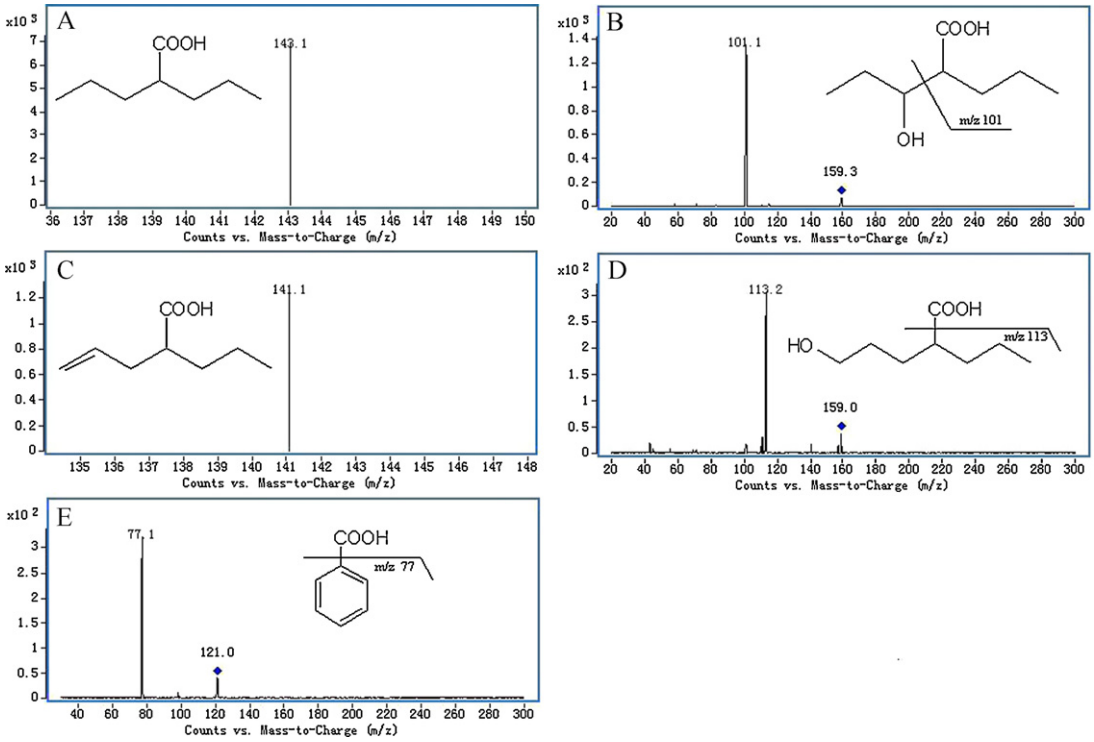

Fig. 1. Chemical structures and full scan product ion of precursor ions of VPA (A), 3-OH-VPA (B), 4-ene-VPA (C), 5-OH-VPA (D) and IS (E).

poor detectability due to the absence of a strong chromophore or fluorophore. HPLC with UV or fluorescence detection, in combination with pre- or post-column chemical derivatization, constitutes a convenient approach to overcome this problem.

Liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS) can quantitatively determine VPA in human plasma [19]. LC–MS/MS has been developed to detect VPA in human plasma [20]. Cheng et al. [21] reported simultaneous determination of VPA and its major metabolite 2-propyl-4-pentenoic acid (4-ene-VPA) in human plasma based on pre-column derivatization using 4-dimethylamino benzylamine dihydrochloride. However, all these methods are time-consuming due to multiple sample preparation/derivatization procedures. It is therefore necessary to develop a rapid, sensitive and convenient method that can simultaneously determine VPA and its metabolites in human plasma.

To our knowledge, there is no study reporting the use of LC-MS method for simultaneous quantitative determination of VPA and its three metabolites (3-OH-VPA, 4-ene-VPA and 5-OH-VPA) in biological samples. To study the pharmacokinetics of VPA, we developed an LC–MS/MS method for simultaneous determination of VPA and its three metabolites, with advantages of high sensitivity, specificity and quickness.

2. Experimental procedures

2.1. Chemicals and reagents

Chemicals and reagents included 2-propylpentanoic acid (VPA, Fig. 1A) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); 3-hydroxy valproic acid (3-OH-VPA, Fig. 1B), 2-propyl-4-pentenoic acid (4-ene-VPA, Fig. 1C) and Rac 5-hydroxy valproic acid sodium (5-OH-VPA, Fig. 1D) (Toronto Research Chemicals Inc., Toronto, Canada); benzoic acid (internal standard, IS, Fig. 1E) (China Medicine (Group) Shanghai Chemical Reagent Corporation, Shanghai, China); HPLC-grade methanol and acetonitrile (Merck Company, Darmstadt, Germany); formic acid and ammonium acetate (Tedia Company, Tedia Fairfield, OH, USA); ultrapure water (Milli-Q Reagent Water System, Millipore, MA, USA); Oasis® HLB 1cc (10 mg) extraction cartridges used for sample preparation (Waters, Milford, Massachusetts, USA); and human blank plasma(plasma from subjects who were not receiving the drug) (Shanghai Red Cross Blood Center, Shanghai, China).

2.2. Liquid chromatography–tandem mass spectrometry

All experiments were carried out on Agilent 1200 series HPLC and interfaced to an Agilent 6410 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Corporation, MA, USA). All data were acquired and analyzed using Agilent 6410 Quantitative Analysis version B.01.02 analyst data processing software (Agilent Corporation, MA, USA). The separation was performed using a ZORBAX SB–C_8 column (3.5 μm, 2.1 × 100 mm, I.D. Agilent Corporation, MA, USA) with an isocratic mobile phase consisting of methanol-10 mM ammonium acetate (80:20, v/v) containing 0.1% formic acid at a flow rate of 0.3 mL/min. The column temperature was maintained at 35 °C. The injection volume was 10 μL and the analysis time was 2.0 min per sample.

VPA and its three metabolites were achieved using electrospray in the negative mode with the spray voltage set at 4000 V. Nitrogen was used as nebulizer gas, and nebulizer pressure was set at 40 psi with a source temperature of 105 °C. Desolvation gas (nitrogen) was heated to 350 °C and delivered at a flow rate of 10 L/min. For collision-induced dissociation (CID), high-purity nitrogen was used as collision gas at a pressure about 0.1 MPa. Quantitation was performed in the multiple reaction monitoring (MRM) mode. Table 1 shows the optimized MRM parameters for detected components. The peak widths of precursor and product ions were maintained at 0.7 amu at half-height in the MRM mode.

Table 1
Optimized MRM parameters for VPA, 3-OH-VPA, 4-ene-VPA, 5-OH-VPA and IS.

Component	Precursor ion	Fragmentor energy (V)	Collision energy (eV)	Product ion
VPA	143	100	0	143
3-OH-VPA	159	100	14	101
4-ene-VPA	141	80	0	141
5-OH-VPA	159	100	20	113
Benzoic acid (IS)	121	80	14	77

2.3. Preparation of standard and quality control (QC) samples

The standard stock solutions of VPA, 3-OH-VPA, 4-ene-VPA, 5-OH-VPA and IS were prepared in methanol to a final concentration of 1 mg/mL for each analyte, diluted to seven concentrations for construction of calibration plots in the range of 2.03–152.25 μg/mL, 51.5–1030, 50.15–5015 and 51.25–1025 ng/mL, respectively. Internal standard working solution (2.5 μg/mL) was prepared by diluting the stock solution of benzoic acid with water. All the working solutions were kept at 4 °C.

Quality control samples for VPA, 3-OH-VPA, 4-ene-VPA and 5-OH-VPA were made up in plasma by an independent analyst using a new stock solution, at three-level concentrations of 5, 20 and 100 μg/mL for VPA, 100, 400 and 800 ng/mL for 3-OH-VPA, 100, 500 and 2000 ng/mL for 4-ene-VPA, 100, 400 and 800 ng/mL for 5-OH-VPA, respectively. The QC samples were stored at −20 °C and brought to room temperature (25 °C) before being processed together with the clinical samples.

2.4. Sample preparation

A plasma sample (0.2 mL) was pipetted into a microcentrifuge tube, with addition of 20 μL IS working solution (2.5 μg/mL) and 50 μL hydrochloric acid (10%, v/v). The mixture was vortex-mixed for 30 s and loaded onto an Oasis® HLB SPE cartridge (10 mg, Waters Co., Milford, USA), which had been conditioned by washing with methanol (2 mL) followed by water (2 mL). The Oasis cartridge was rinsed with water (2 mL) followed by 10% methanol (1 mL), and then eluted with 90% methanol (1 mL). Then, the elutant was transferred into an injector vial, and a 10 μL aliquot was injected into the chromatographic system.

2.5. Human sample collection

Ninety-five epileptic patients (30 women and 65 men), the means (ranges) of age and body weight were 34.6 (25–45) years and 62.0 (51–72) kg, respectively. The experimental method was reviewed and approved by the Ethics Committee of Changzheng Hospital, and performed in Changzheng Hospital, Shanghai, China. Ninety-five epileptic patients took VPA (Sanofi-aventis (hangzhou) Pharmaceuticals Co., Ltd.) at a dose of 500 mg twice daily for more than one month, and then venous blood samples (3 mL each) were drawn from the patients at 12 h after the last dose. Plasma samples were immediately separated by centrifugation at 2000 × *g* for 10 min at 4 °C. All samples were stored at −80 °C until analysis.

2.6. Assay validation

Comparison of the chromatograms of the blank and the spiked human plasma was used to assay the selectivity of the method. To develop a reliable and reproducible method, the matrix effect was also investigated. The matrix effects are generally due to the influence of coeluting, undetected matrix components reducing or enhancing the ion intensity of the analytes and affect the reproducibility and accuracy of the assay. Five different batches of drug-free plasma from healthy volunteers were processed according to the described sample preparation. The absolute matrix effect of the plasma on ionization efficiency was assessed by comparing the peak areas of the analytes spiked in extracted blank plasma samples with that of the neat standards at corresponding concentrations. The relative matrix effects between the five batches were measured by calculating the variability in the values. Three different concentrations of VPA (5, 20 and 100 μg/mL), 3-OH-VPA and 5-OH-VPA (100, 400 and 800 ng/mL) and 4-ene-VPA (100, 500 and 2000 ng/mL) were evaluated by analyzing five samples at each level. The same evaluation was perform for IS (2.5 μg/mL).

Extraction recovery of VPA and its three metabolites was determined by comparing the peak area of the QC with that of the corresponding standard solution spiked in extracted blank plasma. Recovery of IS was also determined using the same method.

Standard curves of four concentrations of VPA, 3-OH-VPA, 4-ene-VPA and 5-OH-VPA ranged from 2.03 to 152.25 μg/mL, 51.5 to 1030, 50.15 to 5015 and 51.25 to 1025 ng/mL, respectively. Calibration curves were constructed from the peak area ratio of each analyte to IS versus plasma concentrations using a $1/\chi^2$ weighted linear least-squares regression model.

Six replicates of QC samples at three levels of VPA, 3-OH-VPA, 4-ene-VPA and 5-OH-VPA were included in each run to determine the intra-day and inter-day precision of the assay. The accuracy was determined as the percentage difference between the mean detected concentrations and nominal concentrations. The lower limit of quantification (LLOQ) is defined as the lowest concentration of the standard that can be measured with an acceptable accuracy and precision (≤20% for both parameters).

The stability of three analytes in plasma was assessed by analyzing triplicate QC samples stored for 6 h at ambient temperature, three cycles of freezing at −20 °C and thawing, reconstituted extract at room temperature for 24 h, and stored for 3 month at −20 °C. The concentrations following storage were compared with freshly prepared samples of the same concentrations.

3. Results and discussion

3.1. Optimization of chromatographic and MS/MS conditions

This analytical method was developed and validated for assaying VPA in the therapeutic concentration range for analysis of routine samples. Most reported methods for quantification of VPA concentrations in human plasma are based on GC–MS. In this laboratory we find it easier and more convenient to develop and validate a new LC–MS or LC–MS/MS method using solid-phase extraction (SPE) rather than using GC–MS. To achieve this aim, we optimized the sample extraction procedure, liquid chromatography conditions and MS detection parameters. During development, MS parameters in both positive and negative ionization modes were tuned for VPA, 3-OH-VPA, 4-ene-VPA, 5-OH-VPA and benzoic acid (IS). However, it was found that the response was much higher in the negative ionization mode for VPA compared with that in the positive mode due to its acidic nature. Use of ammonium acetate in the mobile phase to increase the pH of buffer further enhanced the response of VPA and its three metabolites. Being a small aliphatic molecule, VPA and 4-ene-VPA did not give stable product ions at very low or high collision energy (CE). Thus initially, setting of the MS parameters for the detection of VPA and 4-ene-VPA in pseudo MRM mode was done with the aim to get best specificity with respect to any other available matrix ion with *m*/*z* of 143 and 141 by keeping CE at 0 V. For this, the first quadrupole (Q1) was given a filter for scanning only the molecular ion with *m*/*z* of 143 and 141 with unit resolution and keeping the third quadrupole (Q3) filter to scan the (false) product molecular ion with *m*/*z* of 143 and 141 with low resolution. Thus, the MS/MS fragmentation of VPA and

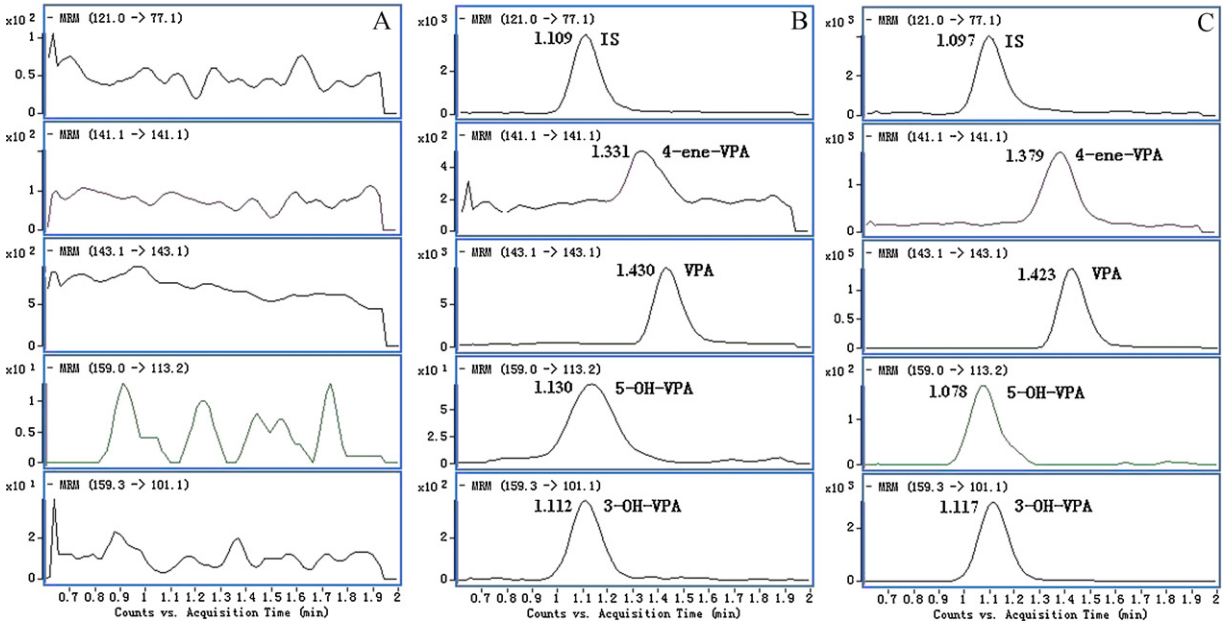

Fig. 2. Representative MRM chromatograms of VPA, 3-OH-VPA, 4-ene-VPA, 5-OH-VPA and IS in human plasma. (A) A blank plasma sample; (B) a blank plasma sample spiked with VPA and its three metabolites at the lower limit of quantification; (C) test plasma sample.

4-ene-VPA did not yield any suitably abundant and characteristic fragment ions for use in MRM, which forced us to choose single ion monitoring (SIM) only.

To get the richest relative abundance of precursor ions and product ions, the parameters for fragmentor energy and collision energy were optimized, and the MRM transition was chosen to be m/z 143 → 143 for VPA, m/z 159 → 101 for 3-OH-VPA, m/z 141 → 141 for 4-ene-VPA, m/z → 113 for 5-OH-VPA and m/z 121 → 77 for IS. Fig. 1 shows the spectra of full scan product ion of precursor ions of the four analytes and IS.

3.2. Sample pre-treatment

Due to the complex nature of plasma, a sample pre-treatment is often needed to remove protein and potential interferences prior to LC–MS/MS analysis. Liquid–liquid extraction with different organic solvents and protein precipitation with acetonitrile were evaluated as sample pre-treatment techniques in our earlier study. However, these techniques resulted in strong interferences from the sample matrix and low recoveries of both analyte and IS. Solid phase extraction has been demonstrated as an effective sample pre-treatment procedure to remove protein and potential interfering endogenous components in plasma. By changing solvent materials and solvent composition selectivity can be optimized. Therefore, SPE was selected as sample pre-treatment technique. Several solid-phase packing materials from various manufacturers such as Oasis® HLB SPE, Agilent SAMPLIQ C18 (1 mL, 100 mg) and Agela Cleanert ODS C18 (1 mL, 100 mg) were tested for the present application, of which Oasis® HLB SPE cartridge was found to meet the criteria of clean injection extracts and high recovery (>86%). Prior to SPE, pH was adjusted to 2.5 with 10% hydrochloric acid. At this pH both VPA and IS will be completely protonated and adsorption on the HLB cartridge is enhanced. At pH 7.4, both VPA and IS are fully ionized and are not adsorbed on the HLB cartridge, whereas, at pH 4.5, VPA and IS are partly ionized and some degree of adsorption is observed. To increase the throughput, it is necessary to avoid the evaporation and reconstitution step in the sample preparation. So we tried direct injection of an aliquot of the eluted 100% methanol solution into the chromatographic system, but the peak shapes were not satisfactory. Different ratios of methanol and water were tried as an elution solvent to obtain good peak shapes, and 90% methanol met this criterion.

3.3. Method validation

3.3.1. Assay selectivity and matrix effect

LC–MS/MS has high selectivity because only selected ions produced from selected precursor ions are monitored. Comparison of the chromatograms of the blank and the spiked human plasma (see Fig. 2) indicated no significant interference at the retention times of the analytes and the IS.

The possibility of a matrix effect caused by ionization competition between the analytes and co-eluents exists when LC–MS/MS is used for analysis. To evaluate the matrix effect in the experiment, chromatographic peak areas of each analyte from the spike-after-extraction samples at low and high concentrations were compared with the neat standards at the same concentrations. The percent nominal concentrations estimated were within the acceptable limits (89.5–119.8%) after evaluation of nine different lots of plasma. The same evaluation was performed on IS, and no significant differences in peak area were observed. Thus, ion suppression or enhancement from plasma matrix was negligible for this method.

3.3.2. Linearity of calibration curves and lower limit of quantification (LLOQ)

Calibration curves were constructed by plotting the peak area ratios (analyte/IS) of plasma standards versus nominal concentration. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighing factors ($1/\chi$, $1/\chi^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/\chi^2$ weighing factor, giving a mean linear regression equation for the calibration curve. The standard calibration curve for VPA, 3-OH-VPA, 4-ene-VPA and 5-OH-VPA in spiked human plasma were linear over the ranges 2.03–152.25 μg/mL, 51.5–1030, 50.15–5015 and 51.25–1025 ng/mL, respectively. The squares of the linear correlation coefficients were all over 0.995. Typical equations for the calibration curve were as follows:

Table 2
Precision and accuracy for the analytes in human plasma ($n = 15$, 5 replicates per day for 3 days).

Analyte	Concentration added (ng/mL)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
		Measured concentration (mean ± S.D., ng/mL)	Precision (R.S.D.%)	Accuracy (RE%[a])	Measured concentration (mean ± S.D., ng/mL)	Precision (R.S.D.%)	Accuracy (RE%[a])
VPA	5075	4636.31 ± 54.36	1.2	−8.6	4697.45 ± 193.41	4.1	−7.4
	20300	20161.32 ± 69.80	0.4	−0.7	20139.41 ± 660.99	3.3	−0.8
	101500	91753.90 ± 2586.98	2.9	−9.6	92993.45 ± 3183.37	3.4	−8.4
3-OH-VPA	103	89.56 ± 3.41	3.8	−13.1	88.95 ± 5.30	6.0	−13.6
	412	349.78 ± 25.72	7.4	−15.1	357.04 ± 25.66	7.2	−13.3
	824	768.85 ± 31.53	4.1	−6.7	744.17 ± 40.02	5.4	−9.7
4-ene-VPA	100.3	104.22 ± 11.61	11.2	3.9	104.50 ± 8.34	8.0	4.2
	501.5	465.43 ± 41.59	8.9	−7.2	459.56 ± 34.29	7.5	−8.4
	2006	1747.60 ± 81.88	4.7	−12.9	1766.62 ± 81.31	4.6	−11.9
5-OH-VPA	102.5	99.38 ± 14.04	14.1	−3.0	98.12 ± 9.15	9.3	−4.3
	410	382.36 ± 42.29	11.1	−6.7	387.11 ± 41.82	10.8	−5.6
	820	769.95 ± 76.98	10.0	−6.1	794.89 ± 64.73	8.1	−3.1

[a] RE is expressed as (mean measured concentration /spiked concentration^{-1}) × 100%.

VPA	$A = 3.42 \times 10^{-4}\ C - 0.2347$	$r = 0.9987$	LOD 30 ng/mL
3-OH-VPA	$A = 1.60 \times 10^{-3}\ C + 0.0211$	$r = 0.9956$	LOD 10 ng/mL
4-ene-VPA	$A = 2.14 \times 10^{-4}\ C + 0.0286$	$r = 0.9976$	LOD 30 ng/mL
5-OH-VPA	$A = 5.54 \times 10^{-5}\ C + 0.0130$	$r = 0.9962$	LOD 10 ng/mL

where C is the plasma concentration of each analyte (ng/mL) and A is the peak–area ratio of each analyte to IS.

The lower limit of quantification for determination of VPA, 3-OH-VPA, 4-ene-VPA and 5-OH-VPA in plasma, defined as the lowest concentration analyzed with accuracy within ±20% and precision ≤20%, was 2.03 μg/mL, 51.5, 50.15 and 51.25 ng/mL, respectively. These limits are sufficient for clinical drug monitoring of VPA and its three metabolites following an oral administration of VPA.

3.3.3. Assay precision and accuracy

The method showed very good precision and accuracy. Intra-day and inter-day precision was assessed from the results of QCs by one-way analysis of variance (ANOVA). The mean values and R.S.D. for QC samples at three concentration levels were calculated over three validation runs. Five replicates of each QC level were determined in each run. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QCs and expressed as the relative error (R.E.). Table 2 summarizes the intra-day and inter-day precision and accuracy for the four analytes from the QC samples. All intra-day and inter-day precision and accuracy were acceptable.

3.3.4. Extraction recovery

To investigate extraction recovery, a set of samples ($n = 3$ at each concentration in unique lots of plasma) was prepared by spiked VPA and its three metabolites into plasma. Each of the samples was also spiked with IS at the working concentration of 2.5 μg/mL. The samples were subsequently processed using the procedure described previously. The extraction recoveries of the analytes under the solid phase extraction conditions were summarized in Table 3. The recovery of the internal standard was 89.16 ± 6.14% in human plasma.

3.3.5. Analyte stability

The stability of VPA, 3-OH-VPA, 4-ene-VPA and 5-OH-VPA in human plasma and mobile phase were investigated. The analyte was found to be stable in human plasma stored for 1 month at −20 °C and in reconstituted mobile phase at room temperature for 24 h (<5% reduction). After storage at −4 °C for 2 months, no obvious reduction was found in the stock and working solutions. The analyte was found to be stable after three freeze-thaw cycles with a reduction less than 15%. The analytes were also shown to be stable in human plasma at room temperature for at least 12 h with a reduction less than 15% (as shown in Table 4).

Table 3
Extraction recoveries of analytes in human plasma ($n = 5$).

Analyte	Concentration added (ng/mL)	Recovery (mean ± S.D., %)	R.S.D.%
VPA	5075	94.2 ± 4.5	4.8
	20300	94.3 ± 1.8	1.9
	101500	90.8 ± 2.5	2.8
3-OH-VPA	103	95.2 ± 5.2	5.2
	412	97.3 ± 15.5	15.9
	824	92.1 ± 10.7	11.6
4-ene-VPA	100.3	92.2 ± 7.4	6.1
	501.5	95.8 ± 5.8	5.4
	2006	93.8 ± 5.4	5.8
5-OH-VPA	102.5	93.2 ± 3.2	2.7
	410	87.4 ± 10.6	12.1
	820	90.1 ± 6.7	7.4

3.4. Application of plasma clinical samples in epileptic patients

The validated method was successfully applied to determination of plasma concentrations of VPA and its three metabolites after oral administration. Plasma samples from 95 epileptic patients were assayed according to the proposed method. The results are shown in Fig. 3. All patients were on long-term VPA treatment, and thus steady-state VPA and its three metabolites levels were expected.

Table 4
Stability of analytes in human plasma ($n = 5$).

Analyte	Concentration added (ng/mL)	Stability (%RE[a])		
		Three freeze–thaw	Short-term (12 h at room temperature)	Long-term (1 month at −20 °C)
VPA	5075	−1.6	8.0	4.5
	20300	−1.9	−1.5	7.5
	101500	−4.8	6.1	6.3
3-OH-VPA	103	−1.0	4.1	6.8
	412	3.0	−5.1	6.2
	824	12.6	−7.4	5.9
4-ene-VPA	100.3	0.1	3.4	5.3
	501.5	5.8	−1.1	−11.3
	2006	6.8	2.1	5.3
5-OH-VPA	102.5	2.6	4.9	9.3
	410	10.3	8.2	10.6
	820	12.3	9.6	7.3

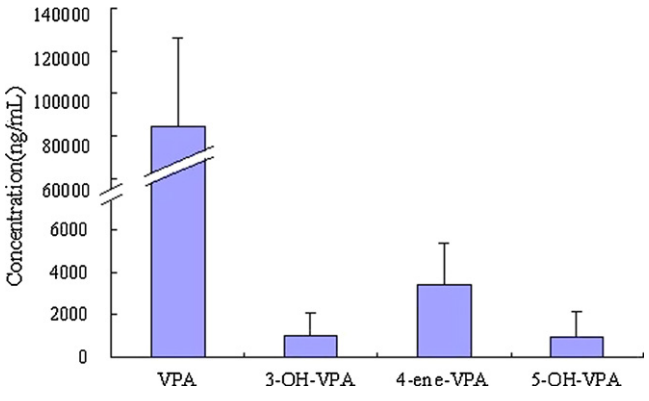

Fig. 3. Mean plasma concentrations of VPA, 3-OH-VPA, 4-ene-VPA and 5-OH-VPA in epileptic patients ($n = 95$) after oral administration of VPA at 500 mg twice daily.

A representative chromatogram obtained from a clinical plasma sample of an epileptic patient is shown in Fig. 2.

4. Conclusion

The analytical method has been successfully applied to assay plasma concentrations of VPA and its three metabolites in human plasma from epileptic patients, with high selectivity, sensitivity, precision and accuracy, and using a small sample volume (200 μL). The method also has the advantage of using SPE to obtain clean and consistent extracts with minimum matrix interference. Because of the relative short chromatographic run time (2.0 min) and straightforward sample pre-treatment procedure, the method is easy to follow and can be adopted for clinical drug monitoring.

References

[1] J.R. Stephens, R.H. Levy, Pharm. Weekbl. Sci. 14 (1992) 118.
[2] R.H. Levy, D.D. Shen, Antiepileptic Drugs (1995) 605.
[3] E. Tanaka, J. Clin. Pharm. Ther. 24 (1999) 87.
[4] H. Nau, W. Löscher, Epilepsia 25 (1984) S14.
[5] A.W. Rettenmeier, W.P. Gordon, H. Barnes, T.A. Baillie, Xenobiotica 17 (1987) 1147.
[6] A.E. Rettie, A.W. Rettenmeier, W.N. Howald, T.A. Baillie, Science 235 (1987) 890.
[7] A.J.M. Sadeque, M.B. Fisher, K.R. Korzekwa, F.J. Gonzalez, A.E. Rettie, J. Pharmacol. Exp. Ther. 283 (1997) 698.
[8] P.C. Ho, F.S. Abbott, U.M. Zanger, T.K.H. Chang, Pharmacogenomics J. 3 (2003) 335.
[9] J.W. Kesterson, G.R. Granneman, J.M. Machinist, Hepatology 4 (1984) 1143.
[10] W. Ehrenthal, M. Rochel, Arzneim-Forsch 32 (1982) 449.
[11] A.S. Wohler, A. Poklis, J. Anal. Toxicol. 21 (1997) 306.
[12] A. Carlin, J. Simmons, J. Chromatogr. B Biomed. Sci. Appl. 696 (1997) 115.
[13] S.V. Gopaul, K. Farrell, F.S. Abbott, J. Mass Spectrom. 35 (2000) 698.
[14] M.R. Anari, R.W. Burton, S. Gopaul, F.S. Abbott, J. Chromatogr. B Biomed. Sci. Appl. 742 (2000) 217.
[15] G. Nagai, S. Ono, N. Yasui-Furukori, A. Nakamura, K. Mihara, T. Kondo, Ther. Drug Monit. 31 (2009) 592.
[16] V. Pucci, R. Mandrioli, M.A. Raggi, Electrophoresis 24 (2003) 2076.
[17] J.H. Wolf, L. Veenma-van der Duin, J. Korf, J. Chromatogr. 487 (1989) 496.
[18] M.C. Lin, H.S. Kou, C.C. Chen, S.M. Wu, H.L. Wu, J. Chromatogr. B 810 (2004) 169.
[19] N.V. Ramakrishna, K.N. Vishwottam, S. Manoj, M. Koteshwara, M. Santosh, J. Chidambara, B.R. Kumar, Rapid Commun. Mass Spectrom. 19 (2005) 1970.
[20] D.S. Jain, G. Subbaiah, M. Sanyal, P. Shrivastav, Talanta 72 (2007) 80.
[21] H. Cheng, Z.F. Liu, W. Blum, J.C. Byrd, R. Klisovic, M.R. Grever, G. Marcucci, K.K. Chan, J. Chromatogr. B 850 (2007) 206.