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ORIGINAL ARTICLE

Development and Validation of an LC/MS/MS Method for Determination of Valproic Acid and Its Metabolite 2-Propyl-4-pentenoic Acid in Monkey Plasma

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Abstract A rapid, accurate, and sensitive liquid chromatography/mass spectrometry (MS)/MS method for the quantitative determination of valproic acid (VPA) and its metabolite, 2-propyl-4-pentenoic acid (4-ene VPA), in monkey plasma was developed and validated. The sample extraction was performed using hydrophilic-lipophilic balance cartridge. The analytes were separated on a Kinetex C18 (2.1 mm × 100 mm, 2.6 μm) analytical column under a mobile phase consisting of 10 mM ammonium formate (pH 8.0)/methanol (20/80, v/v) and isocratic flow at 0.15 mL/min. The tandem mass spectrometer was operated in negative electrospray ionization with selected ion monitoring conditions, 143.0, 141.0, and 121.0 for VPA, 4-ene VPA, and benzoic acid (internal standard), respectively. The linearity of calibration curve ranging from 0.1 to 20 μg/mL was at least 0.9996 (coefficient of correlation, r) for both analytes. Intra- and inter-day precisions for both analytes were lower than 15%, resulting from quality control (QC) samples at concentration of 0.2 (low QC), 1.6 (middle QC), and 16 (high QC) μg/mL except at the lower limit of quantification (LLOQ) (0.1 μg/mL) level, which was less than 20%. The intra- and inter-day accuracies were within ±15%. The recoveries were 84.4–90.8% for VPA and 88.2–100.6% for 4-ene VPA. Both analytes were stable throughout short-term temperature, post preparation for 24 h, and three freeze/thaw cycles, validating that this method could be applied to toxicokinetic and pharmacokinetic studies.

Keywords liquid chromatography/mass spectrometry (MS)/MS · method validation · 2-propyl-4-pentenoic acid · valproic acid

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Introduction

Valproic acid (VPA), an established antiepileptic drug with a simple chemical structure, is used in the treatment of primary generalized, partial, and myoclonic seizures (Willmore, 2003). Metabolism of VPA is very complex and is the result of hepatic microsomal oxidation and glucuronidation (Davis et al., 1994). Various metabolites have been identified. Although the mechanism is not fully elucidated, reactive VPA metabolites such as 4-ene VPA and 2,4-diene VPA may be related to the hepatoxicity of VPA (Zimmerman and Ishak, 1982; Granneman et al., 1984; Rettenmeier et al., 1986). Therefore, establishing a sensitive and accurate analytical method is important in monitoring the levels of VPA and its metabolite for the toxicokinetic and pharmacokinetic studies.

Several methods for the quantification of VPA in biological matrix have been described. Due to chemical structure properties such as the absence of a strong chromophore or fluorophore, the derivatization process to provide a suitable sensitivity is required for high-performance liquid chromatography (HPLC) analysis with UV or fluorescence detection (Nakamura et al., 1984; Vam der Horst et al., 1988; Wolf et al., 1989; Lucarelli et al., 1992). However, these methods lack sensitivity and require a laborious and time-consuming sample preparation. Recently, several liquid chromatography/mass spectrometry (LC/MS) methods have been reported (Pucci et al., 2005; Ramakrishna et al., 2005; Jain et al., 2007; Matsuura et al., 2008). These methods provide enhanced sensitivity without chemical modification. Ramakrishna et al. (2005) developed an LC/MS/MS method for determination of VPA in 200 μL of human plasma to achieve a lower limit of quantification of 0.5 μg/mL in solid phase extraction (SPE) sample preparation. Also, protein precipitation (PPT) method using 96-well plate was established to determine VPA in mouse plasma (Pucci et al., 2005). However, only few methods for the

simultaneous determination of VPA and it metabolites have been reported (Cheng et al., 2007; Amini-Shirazi et al., 2010) with LC/MS/MS and gas chromatography (GC)/MS. Even though the GC/MS method has been employed in N,O-bis(trimethylsily) trifluoroacetamide (BSTFA) derivatization (Amini-Shirazi et al., 2010), this method lacks the sensitivity of VPA compared to that of its metabolite.

To support the toxicokinetic and pharmacokinetic studies, a sensitive analytical method for the simultaneous determination of VPA and 4-enes VPA in monkey plasma was required. In the present study, a simple, fast and sensitive LC-MS/MS method was developed for the detection with a lower limit of quantification (LLOQ) of 0.1 μg/mL for VPA and 4-ene VPA in 100 μL of monkey plasma. This paper describes the development of the LC-MS/MS method and its validation process.

Materials and Methods

Chemicals and reagents. Sodium VPA, its metabolite, 2-propyl-4-pentenoic acid and benzoic acid (internal standard) were obtained from Sigma (St. Louis, MO) (Fig. 1). HPLC-grade acetonitrile and methanol were purchased from Burdick & Jackson (Ulsan, Korea). Ammonium formate was obtained from Aldrich (St. Louis, MO). Hydrochloric acid and ammonium solution were purchased from Sigma and Merck (Darmstadt, Germany), respectively. Distilled water was purified using a Millipore water purification system (Millipore, Molshem, France). Oasis HLB extraction cartridges (30 mg, 1 mL) were from Waters (Milford, MA).

Chromatographic methods. The experiments were carried out with the 1200 system (Agilent Technology, Waldbronn, Germany), which consisted of a G1312A binary pump, a G1379B degassing unit, a G1367B autosampler equipped with a G1330B thermostat, and a G1316A thermostatted column compartment. The stationary phase was a Kinetex® C18 column (2.6 μm, 100 mm × 2.1 mm i.d., Phenomenex, Torrance, CA at 30°C). The chromatographic separation was achieved using a mobile phase consisting of 10 mM ammonium formate buffer (pH 8.0 adjusted with ammonium hydroxide) and methanol (20:80, v/v), with the flow rate set at 0.15 mL/min. The column effluent was connected to an electrospray ionization MS interface without splitting. Mass spectrometric detection was performed using an API 5000 triple-quadrupled instrument (MDS-SCIEX, Toronto, Canada) in selected ion monitoring (SIM) mode. A turbo-electrospray interface in negative ionization mode was used. The ion transitions were monitored at *m/z* 143.0 143.0 for VPA, *m/z* 141.0 141.0 for 4-ene VPA, and *m/z* 121.0 121.0 for benzoic acid (IS). The Analyst Software Version 1.5 package supplied by Applied Biosystems (MDS SCIEX, Foster city, CA) was used for LC/MS/MS control and data acquisition and processing. The main parameters of the mass spectrometer are summarized in Table 1.

Table 1 MS parameters for valproic acid, 4-ene valproic acid, and benzoic acid (IS)

Parameters	Valproic acid	4-ene Valproic acid	Benzoic acid (IS)
Declustering Potential (DP, V)	–50	–115	–15
Entrance Potential (EP, V)	–10	–10	–10
Collision Energy (CP, V)	–21	–10	–8
Collision Cell Exit Potential (CXP, V)	–15	–15	–15
Dwell time	300	300	300

Preparation of calibration standard and quality-controlled samples. Stock solutions of 2 mg/mL each VPA and 4-ene VPA and 1 mg/mL IS were prepared by dissolving requisite amount of 50% methanol and were stored at –20°C. Working standard solutions were prepared over a range of 1–200 μg/mL by serial dilution of the stock solution with 50% methanol and then stored at –20°C. Plasma calibration standards of 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/mL were prepared by mixing the working standard with blank monkey plasma. Quality-controlled (QC) samples were prepared from an independent stock solution at concentrations of 0.1 for LLOQ, 0.2 for low quality control (LQC), 1.6 for middle quality control (MQC), and 16 μg/mL for high quality control (HQC) by dilution of the working stock solution with blank monkey plasma. The IS working solution (10 μg/mL) was prepared by diluting its stock solution with 50% methanol.

Sample preparation. Calibration standards and QC samples were acidified by adding 100 μL of 10% hydrochloric acid solution (v/v). After spiking 10 μL of IS, the samples were shaken for 5 min and then centrifuged for 10 min. The upper layer was loaded onto an OASIS HLB SPE cartridge (Waters, Milford, MA), which had been preconditioned by washing with 1 mL of methanol followed by 1 mL of water. The SPE cartridge was rinsed with 1 mL of water followed by 1 mL of 5% methanol and then eluted with 500 μL of 75% methanol. An eluate (3 μL) was injected into LC/MS/MS system.

Bioanalytical method validation. The parameters for validation of the method were accuracy, precision, selectivity, linearity,

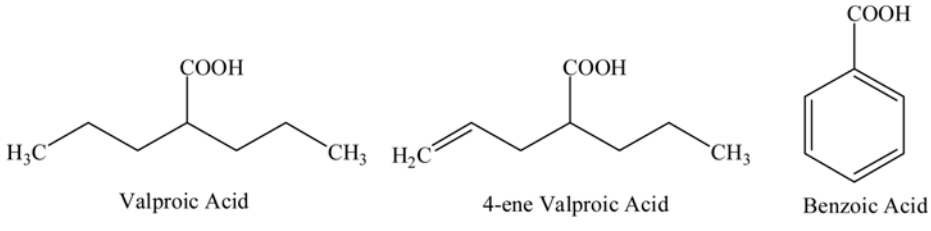

Fig. 1 Structure of valproic acid, 4-ene valproic acid and benzoic acid (IS)

recovery, matrix effect, and stability. These validation experiments were conducted in compliance with FDA guidance for Bioanalytical Method Validation. To evaluate the specificity of the analytical procedure, blank monkey plasma samples obtained from six independent sources were extracted and analyzed for the presence of interfering endogenous substances. The linearity of the standard curve was examined based on the result of calibration curve. The calibration curve consisted of a blank (matrix sample processed without internal standard), a zero (matrix sample processed with internal standard), and six non-zero samples including LLOQ. The regression parameters of slope, intercept, and correlation coefficient were calculated by weighted (1/x) linear regression using the Analyst 1.5. The precision and accuracy of VPA and 4-ene VPA were evaluated by each six determinations for LLOQ, low QC, middle QC, and high QC samples. The inter-batch of precision and accuracy were assessed based on the results of three batches for three different days. The precision and accuracy of the assay were expressed as coefficient of variation (CV, %) and relative error (RE, %), respectively. The matrix effect of VPA and 4-ene VPA in monkey plasma was assessed by comparing the peak areas of the analytes dissolved in mobile phases with those of analytes spiked into plasma extracts, which mean unextracted samples. The recovery was evaluated by comparing the analytical results for extracted samples with unextracted samples that represent 100% recovery. The matrix effect and recovery were assessed with six independent matrices for low, middle, and high QCs. Stability procedures evaluate the stabilities of the analytes during sample collection and handling after long- and short-term storages, after freeze and thaw cycles, and the analytical process. The stock solution stabilities of valproic acid and 4-ene VPA were evaluated by storing at approximately –80°C for 32 days. The stabilities of VPA and 4-ene VPA in monkey plasma were evaluated during three freeze-thaw cycles, at room and freezing temperatures for up to 24 h and 15 days, respectively. Post-preparative stability was assessed after leaving the samples for 24 h in an autosampler. Three aliquots each of low and high QC samples were used for the stability test. To determine toxicokinetic (TK) samples with a higher concentration than upper limit of quantification (ULOQ), the samples having 10 and 100 times higher than HQC sample were prepared, diluted with blank monkey plasma and analyzed.

Results and Discussion

The mass spectra of VPA and 4-ene VPA were acquired by infusion mode. However, no suitable fragment ion was detected for MRM mode during optimization of MS parameters. For this reason, the mass spectrometer was operated in SIM (selected ion mode). The analytes were monitored using SIM of the deprotonated molecules at *m/z* 143 for VPA, 141 for 4-ene VPA, and 121 for benzoic acid as internal standard (IS) for the $[M-H]^-$ ions. Representative chromatograms of blank and spiked monkey plasma samples containing VPA and 4-ene VPA at a concentration of 0.1 μg/mL are shown in S1 of supplementary material. The specificity for the analysis is shown by symmetrical resolution of the peaks, with no significant chromatographic interference around the retention time of the analytes in blank monkey plasma obtained from a total of six independent sources.

The calibration curve was linear over the concentration range of 0.1 to 20 μg/mL of VPA and 4-ene VPA in monkey plasma with correlation coefficients 0.9996 and consistent slope values when evaluated by weighed (1/x) linear regression. For each point on the calibration curves for VPA and 4-ene VPA, the back-calculated concentration from the equation of the regression analysis were always within acceptable limits for accuracy and precision of ±15% (Table 2). The LLOQ for the determination of VPA and 4-ene VPA in monkey plasma were established at 0.1 μg/mL. At this concentration, the values for precision and accuracy did not exceed 20% (Table 2).

Table 3 shows the intra- and inter-batch accuracy and precision data of VPA and 4-ene VPA respectively. The precisions were always less than 15% for VPA and 4-ene VPA, except for LLOQ of 4-ene VPA. However, the % CV value of LLOQ of 4-ene VPA was within 20% of the acceptance criteria. The accuracy was within 15% of the nominal value at the various concentrations analyzed in six determinations for four concentrations.

The matrix effect and recovery were assessed with six independent matrices for low, middle, and high QCs. The means of matrix effects for VPA were 121.7, 112.0, and 108.2% at low, middle, and high QC samples, respectively. For 4-ene VPA, the mean values of matrix effect were 116.0, 112.1, and 108.5% at low,

Table 2 Linearity of valproic acid and 4-ene valproic acid in monkey plasma

Nominal concentration (μg/mL)	Found concentration Mean ± SD (μg/mL)	%CV	%RE
Valproic acid			
0.1	0.106±0.005	4.7	6.3
0.2	0.203±0.003	1.7	1.6
0.5	0.508±0.017	3.3	1.6
1	0.967±0.022	2.3	–3.3
2	1.89±0.04	2.3	–5.3
5	4.88±0.06	1.2	–2.4
10	10.0±0.2	1.6	0.3
20	20.2±0.6	0.1	1.1
4-ene valproic acid			
0.1	0.0977±0.0063	6.5	–2.3
0.2	0.202±0.006	2.9	0.9
0.5	0.525±0.014	2.7	4.9
1	0.996±0.025	2.5	–0.4
2	1.95±0.09	4.7	–2.3
5	4.92±0.08	1.7	–1.5
10	10.0±0.2	1.9	0.5
20	20.0±0.2	0.9	0.2

Table 3 Inter- and intra-batch precision and accuracy of valproic acid and 4-ene valproic acid in monkey plasma

	Nominal concentration (μg/mL)			
	0.1	0.2	1.6	16
Valproic acid				
Accuracy				
Inter-batch (%RE, n=18)	–3.3	–3.5	–7.2	0.9
Intra-batch (%RE, n=6)	–9.8	–7.0	–9.3	2.0
Precision				
Inter-batch (%CV, n=18)	5.3	3.8	2.1	1.7
Intra-batch (%CV, n=6)	3.1	3.4	1.8	2.3
4-ene valproic acid				
Accuracy				
Inter-batch (%RE, n=18)	–6.8	–8.2	–5.8	–0.6
Intra-batch (%RE, n=6)	–13.1	–11.8	–8.0	–2.2
Precision				
Inter-batch (%CV, n=18)	13.1	8.4	2.4	1.8
Intra-batch (%CV, n=6)	16.6	12.8	2.0	1.6

Table 4 Matrix effect and recovery of valproic acid and 4-ene valproic acid in monkey plasma

	Nominal concentration (μg/mL)		
	0.2	1.6	16
Vaproic acid			
Matrix effect (%)	121.7	112.0	108.2
Recovery (%)	90.8	84.4	85.4
4-ene Valproic acid			
Matrix effect (%)	116.0	112.1	108.5
Recovery (%)	100.6	88.2	89.5

middle, and high QC samples, respectively. The recoveries of the analytes were highly consistent and reproducible. Table 4 shows the matrix effect and recovery data of VPA and 4-ene VPA. The variation between the different plasma lots used was not significant different in both analytes, indicating the independent plasma sources do not adversely affect the quantitation.

The stability of the analytes in monkey plasma under different temperature and time conditions, post-preparative conditions as well as their stability in the stock solution were evaluated. The stabilities for the stock solution samples, which were kept at approximately –80°C for 32 days, were 100.5 and 101.8% for VPA a 4-ene VPA, respectively. For short-term stability determination, freezer-stored QC samples were thawed and kept at room temperature. After 24 h, samples were processed and analyzed. The results show that no significant loss of VPA and 4-ene VPA was observed after storage of plasma at room temperature on the bench-top for at least 24 h (Table 5). The stability of post-preparative samples in autosampler was also evaluated. The processed samples were stable up to 24 h at 4°C (Table 5). The freeze-thaw and the long-term stabilities were established. Plasma samples were stable over at least three freeze-thaw cycles prior to analysis and at approximately –80°C for at least 15 day with no significant loss (Table 5).

Table 5 Stability of valproic acid and 4-ene valproic acid in monkey plasma

	Nominal concentration (μg/mL)	Found concentration Mean ± SD (μg/mL)	% change [a)]
Valproic acid			
Initial concentration	0.2	0.205±0.001	-
	16	15.9±0.2	-
Short-term stability (n=3, 24 h)	0.2	0.189±0.001	–7.8
	16	16.4±0.1	3.6
Post-preparative stability (n=3, 24 h)	0.2	0.191±0.004	–6.8
	16	15.9±0.3	0.2
Freeze/Thaw stability (n=3, 3 cycles)	0.2	0.183±0.006	–10.7
	16	16.5±0.2	3.8
Long-term stability (n=3, 15 days)	0.2	0.190±0.006	–7.3
	16	16.3±0.2	2.7
4-ene Valproic acid			
Initial concentration	0.2	0.214±0.002	-
	16	15.6±0.5	-
Short-term stability (n=3, 24 h)	0.2	0.200±0.002	–6.4
	16	16.3±0.5	4.7
Post-preparative stability (n=3, 24 h)	0.2	0.212±0.015	–0.8
	16	15.6±0.3	0.0
Freeze/Thaw stability (n=3, 3 cycles)	0.2	0.217±0.003	1.4
	16	16.4±0.2	5.1
Long-term stability (n=3, 15 days)	0.2	0.183±0.007	–14.3
	16	16.5±0.3	5.6

[a)]% Change=(Mean concentration of stored samples–Mean concentration of initial samples)/Mean concentration of initial samples × 100

Table 6 Dilution effect of valproic acid and 4-ene valproic acid in monkey plasma

Nominal concentration (μg/mL)	Dilution Factor	Theoretical concentration (μg/mL)	Found concentration Mean ± SD (μg/mL)	%RE
Vaproic acid				
160	10	16	17.4±0.3	8.4
1600	100	16	16.5±0.2	3.2
4-ene Valproic acid				
160	10	16	15.6±0.1	–2.3

The dilution effect of the plasma sample was assessed by dilution of QC samples, 160 and 1600 μg/mL, by dilutions of 10 and 100 times with blank monkey plasma (n=6). The %REs for VPA between found and theoretical concentrations by dilutions of 10 and 100 times were 8.4 and 3.2%, respectively (Table 6). The %REs for 4-ene VPA between found and theoretical concentration by 10 times was –2.3% (Table 6).

In the present study, LC/MS/MS assay for quantification of the antiepileptic drug VPA and its metabolite, 4-ene VPA in monkey plasma involving SPE to prepare samples for analysis and chromatography performed under basic conditions with the positive ion detection mode has been developed and validated.

Concentrations of VPA and 4-ene VPA within the range from 0.1 to 20 μg/mL were determined with a 100-μL sample volume. This method has shown acceptable precision and accuracy as well as adequate sensitivity for the quantification of VPA and 4-ene VPA in monkey plasma. The extraction procedure consistently yielded high recoveries, resulting in high processing efficiency for the assay. The two analytes were shown to be stable under all test conditions, including 15-day storage in a freezer and three freeze-thaw cycles for 24 h at room temperature and 24 h in autosampler after sample preparation. The method was validated according to the US Food and Drug Administration bioanalytical guidance and met the acceptance criteria. The described method was simple, rapid, reliable, reproducible, and sufficiently sensitive for application in toxicokinetic and pharmacokinetic studies.

Supplementary Material

Representative chromatograms of blank monkey plasma spiked with benzoic acid alone and with 0.1 μg/mL of valproic acid and 4-ene valproic acid.

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