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Simultaneous Determination of Valproic Acid and its Toxic Metabolites, 4-ene-VPA and 2,4-diene-VPA in Rat Plasma using a Gas Chromatographic-mass Spectrometric Method

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ABSTRACT – A gas chromatographic-mass spectrometric (GC-MS) method was developed for the simultaneous determination of valproic acid (VPA) and its toxic metabolites, 4-ene-VPA and 2,4-diene-VPA in rat plasma. Extraction was performed in weak acidic condition (pH 5.2) to avoid degradation of 4-ene-VPA and 2,4-diene-VPA. The recoveries for 4-ene-VPA and 2,4-diene-VPA were more than 70% and that for VPA was 33-42%. R value for each compounds exceeded 0.998 in calibration curve during all the analysis. Accuracy and precision ranged from 88.3 to 113.2% and from 2.16 to 14.2%, respectively The method was successfully applied to monitor plasma concentrations of VPA, 4-ene-VPA and 2,4-diene-VPA after intravenous administration of VPA at the dose of 100 mg/kg, suggesting that these toxic metabolites may involved in the hepatotoxicity induced by VPA.

Key words – valproic acid (VPA), 4-ene-VPA, 2,4-diene-VPA, quantitative analysis, GC-MS (gas chromatographic-mass spectrometry), pharmacokinetics

Valproic acid (VPA, 2-propylpentanoic acid, Fig. 1) is a broad-spectrum antiepileptic agent that has a branched-chain carboxylic acid structure. VPA has been used to treat bipolar disorder, migraine, and neuropathic pain (Loscher et al., 1993; Murakami et al., 1992; Sugimoto et al., 1983), but it has been demonstrated to be associated with fatal liver toxicity (Perucca, 2002; Zimmerman et al., 1982; Zafrani et al., 1982; Bryant et al., 1986). Although the mechanism by which VPA leads to liver toxicity has not been fully elucidated, a large body of evidence suggests that reactive VPA metabolites (4-ene-VPA; 2-propyl-4-pentanoic acid and its subsequent metabolite, 2,4-diene-VPA; 2-propyl-2,4-pentanoic acid) may mediate the hepatotoxicity by inhibiting mitochondrial fatty acid β-oxidation (Zimmerman et al., 1982; Gerber et al., 1979; Kassahun et al., 1991). Therefore it is important to monitor the plasma concentration of 4-ene-VPA and 2,4-diene-VPA as well as VPA itself to evaluate effect and toxicity of VPA.

In order to monitor plasma concentration of VPA and its toxic metabolites, it is necessary to have reliable and sensitive method for the simultaneous quantitative determination of those compounds. Because of structural similarity between fatty acid (non polar compound) and VPA, most of analytical method for VPA and/or its metabolite has been used GC-MS (gas chromatography-mass spectrometry) (Fisher et al., 1992; Yu et al., 1995; Leis et al., 2003). But those reports did not focus on the toxic metabolites, 4-ene-VPA and 2,4-diene-VPA, so it is very hard to evaluate pharmacokinetics of VPA and its toxic metabolites. Recently the method using HPLC-MS/MS (high performance liquid chromatography-tandem mass spectrometry) was reported to analyze VPA, however this method was developed only for the quantification of VPA and 4-ene-VPA. Thus it is also not adequate to monitor plasma concentrations of VPA and its toxic metabolites (Cheng et al., 2007). Moreover, sample extraction with organic solvent was performed in the pH condition less than pH 3, which was not favorable condition for 4-ene-VPA and 2,4-diene-VPA since they could be degraded in acidic condition.

In this study we developed adequate method for the quantitative analysis of VPA, 4-ene-VPA and 2,4-diene-VPA in rat plasma. We used mild pH condition to prevent degradation of the metabolites. This analytical method was validated and applied to evaluate pharmacokinetics of VPA and its metabolites after intravenous administration to rats.

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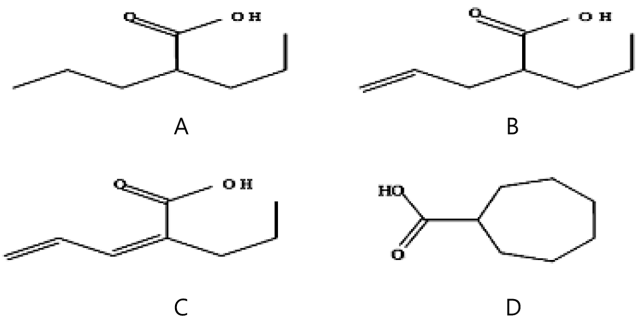

Figure 1. Chemical structures for A: valproic acid (VPA, A), 4-ene-VPA (B), 2,4-diene-VPA (C) and internal standard (IS: cycloheptanecarboxilic acid, D).

Table I. *Characteristic Ions and Quantitative Ions for the Analysis of Cycloheptanecarboxylic Acid, VPA, 4-ene-VPA and 2,4-diene-VPA*

Compounds	Characteristic ions		
	Ion-1	Ion-2	Ion-3
Cycloheptanecarboxylic acid (ISTD)	**199**	155	117
Valproic acid (VPA)	**145**	174	143
4-ene-VPA	**199**	172	185
2,4-diene-VPA	**197**	122	182

*Bold ions were used as the quantitative ion for their respective compounds

Experimental

Materials

Valproic acid (VPA), cycloheptane carboxylic acid (internal standard, IS), ethyl acetate and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Methanol was purchased from Burdick & Jackson (Muskegon, MI, USA). Synthesized 4-ene-VPA and 2,4-diene-VPA were kindly supplied from Bioactive Molecules Center in Korea Institute of Science and Technology (Seoul, Korea).

Apparatus and Instrumental Conditions

An Agilent 6890 series gas chromatography system directly connected with an Agilent 5975 series mass selective detector was used. Samples were injected into a fused-silica capillary column coated with cross-linked methyl silicone (Ultra-1, 25 m×0.2 mm inner diameter, 0.33 μm film thickness) by an Agilent 7683 B series auto-sampler. Helium was used as a carrier gas at a constant flow rate of 0.9 mL/min. The inlet temperature was 280°C, and the split ratio was 10:1. The oven temperature was controlled as follows: the initial temperature was 80°C and it was maintained for 2 min. Then the temperature was raised to 160°C at a rate of 10°C per min. Finally, the temperature was increased to 250°C at a rate of 30°C per min and it was maintained for 2 min. The ion source and detector temperatures were 150°C and 230°C, respectively. The auxiliary temperature was 300°C. The electron impact (EI) ionization mode was used, and all of the ions were monitored in the selected ion monitoring (SIM) mode. All compound has $[M\text{-}CH3]^+$ ion as the highest intensity ion which was used for quantitative analysis. Gas chromatographic and mass spectrometric details (retention time, quantitative ions, qualifier ions, etc.) for all analyzed compounds are given in Table I.

Calibration and QC Samples

A stock solution for each analyte was prepared at a concentration of 1 mg/mL by dissolving in methanol. All the compounds were diluted in methanol to be a concentration range from 0.01 to 100 μg/mL for the generation of calibration curves and method validation. All stock solutions were then stored at -20°C.

Calibration factors were calculated according to a least-squares linear regression. The analytical recovery was determined by comparing the response before and after extraction. Quality control (QC) samples were prepared at low (10 ng/mL for VPA, 0.02 ng/mL for 4-ene-VPA and 2,4-diene-VPA), medium (50 ng/mL VPA, 0.1 ng/mL for 4-ene-VPA and 2,4-diene-VPA) and high (250 ng/mL VPA, 0.5 ng/ml for 4-ene-VPA and 2,4-diene-VPA) concentrations for each compound by spiking an appropriate amount in blank rat plasma.

Sample Preparation

A total 20 μL of cycloheptane carboxylic acid (IS, 10 μg/mL) was added to 100 μL of rat plasma. Following the addition of 1.5 mL of sodium acetate buffer (0.2 M, pH 5.2), liquid-liquid extraction was performed twice with 4 mL of ethyl acetate. The organic phase was separated by centrifugation at 2500 rpm for 5 min, and the supernatant was collected after freezing (-25°C) for 5 min. The supernatant was then dried under a gentle stream of nitrogen at 37°C and kept in a vacuum desiccator with P_2O_5/KOH for at least 60 min. A total 40 μL of MSTFA was added in the residue. Then it was allowed to react at 60°C for 30 min. A final 2 μL of derivatized sample was subjected to GC/MS analysis.

Recovery and Assay Validation

Recovery of each compound was checked with QC samples for VPA, 4-ene-VPA and 2,4-diene-VPA. The recovery was calculated by comparing the absolute peak area of each analyte

after extraction (A) to that before extraction (B); accordingly, recovery = A/B×100 (%). The experimental precision was calculated from the ratio of the standard deviation to the mean and expressed as the coefficient of variation (% CV). The accuracy of the method was determined by the ratio of the concentrations of QC samples to the theoretical concentrations and expressed as percentage. For the validation, QC samples were analyzed on three different days. The LOQ (limit of quantification) was defined as the concentration at which both the relative standard deviation (RSD) and the percentage deviation from the nominal concentration were less than 20%.

Animal Treatment

Six- to eight-week-old, male Sprague-Dawley rats (weighing 280-325 g) were purchased from Orientbio Korea (Seoul, Korea) and were housed in a room with an ambient temperature of 20-23ºC, 12 h light (07:00-19:00) and dark (19:00-07:00) cycles, and a relative humidity of 50±5%. Rats were given a normal standard chow diet (Samtaco Inc, Seoul, Korea) and tap water ad libitum.

Intravenous Administration of Valproic Acid and Sample Collections

Sodium valproate was dissolved in water (10-250 mg/mL) and injected in the doses of 100 mg (as the base)/kg/day (n=5) through carotid vein after cannulation. Blood samples were collected from the carotid artery at 0 (blank), 1, 10, 30 (min), 1, 2, 3, 4, 6, 8, 12, 18, and 24 (hr) after drug administration. The blood samples were centrifuged and the plasma was collected. The plasma samples were then stored at -20ºC before analysis.

Pharmacokinetic Analysis

Terminal elimination constants (β) for valproic acid following intravenous administration were obtained by linear regression of the terminal linear portion of the log plasma concentration-time curves. The corresponding half-life was calculated be 0.693/β. The area under the plasma concentration versus time curve (AUC) from 0 time to infinity was calculated by standard trapezoidal method. All pharmacokinetic parameters were calculated by model independent method.

Results and Discussion

GC-MS Analysis

The GC oven temperature was programmed to generate well separated and evenly shaped peaks for VPA, 4-ene-VPA and 2,4-diene-VPA. The total ion chromatogram for the TMS derivative of each compound is shown in the Fig. 2. As shown

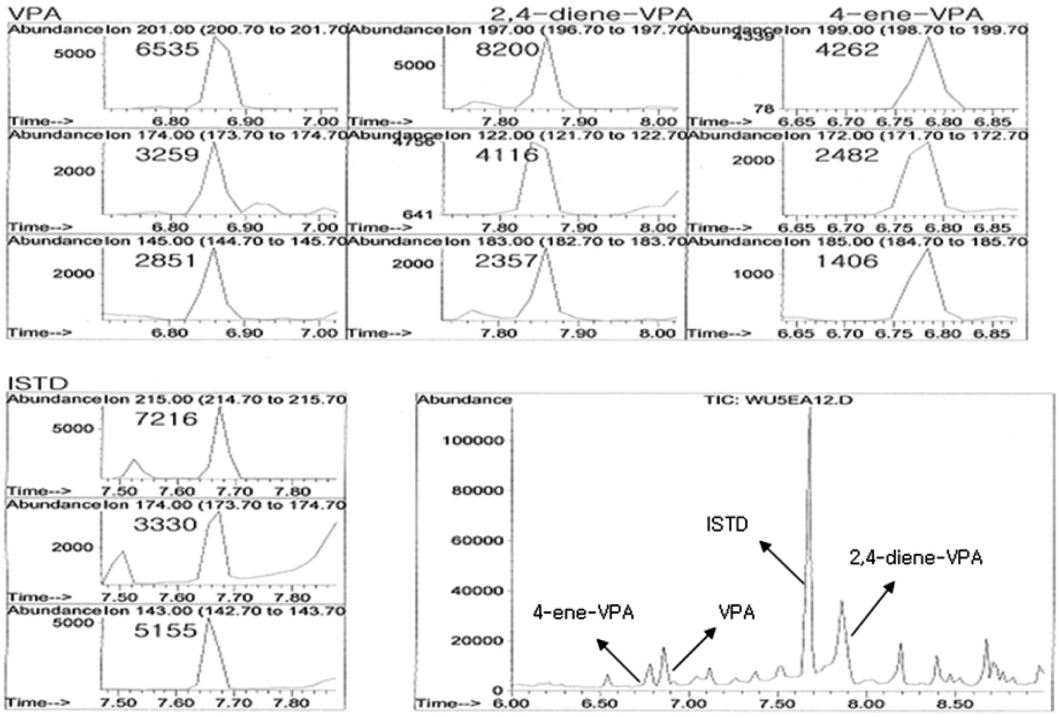

Figure 2. Total and extracted ion chromatogram in SIM (selected ion monitoring) mode for valproic acid (VPA), 4-ene-VPA, 2,4-diene-VPA and internal standard (IS, cycloheptane carboxylic acid). Three columns below the name of compounds shows extracted ion chromatograms of characteristics ions for each compounds and the box in the right bottom shows total ion chromatogram.

in Fig. 2, all compounds were identified with 3 characteristic ions for each compound.

Recovery and Assay Validation

VPA and its metabolite 4-ene-VPA and 2,4-diene-VPA have very different chemical properties such as polarity and the plasma concentration range between the compounds were very diverse (e.g, plasma concentration ranged 1-600 μg/mL for VPA but less than 1 μg/mL for 4-ene-VPA and 2,4-diene-VPA). Thus there were few reports on the simultaneous quantitative determination method for VPA and its toxic metabolite; 4-ene-VPA and 2,4-diene-VPA. However simultaneous determination method for both VPA (parent drug) and toxic metabolites were necessary to evaluate toxicokinetics. Therefore we tried to develop simultaneous quantitative analytical method for VPA and its toxic metabolite. Several organic solvent and pH conditions were tested to increase recovery as well as avoid degradation of metabolites. Strong acidic or alkali condition in the extraction was not favorable since those metabolites could be degradable, so weak acidic, alkalic and neutral pH were tested. The recoveries for the liquid-liquid extraction with diethyl ether, ethyl acetate and the mixture of diethyl ether and ethyl acetate (1:1, v/v) in pH 5.2 (acetate buffer),7.0 (phosphate buffer) and pH 9.0 (carbonate buffer) were examined, respectiverly. From the results of recovery, liquid-liquid extraction with ethyl acetate in pH 5.2 showed better recoveries for all three compounds than any other organic solvents and pH conditions.

The percent recovery for 4-ene-VPA and 2,4-diene-VPA ranged from 70 to 80 (Table II). The pH for the liquid-liquid extraction was weak acidic condition (pH 5.2), thus the recovery for toxic metabolites was quite satisfied. But the recovery of VPA was less than 42%, though VPA concentrations in plasma was much higher than 4-ene-VPA and 2,4-diene-VPA. Therefore, extraction solvent was more optimized for toxic metabolites rather than parent drug, which may be the cause of low recovery of VPA. In spite of low recovery for VPA, there was no problem in the determination of plasma concentrations for VPA. VPA had a LOQ at a concentration of 1 ng/mL, while the LQQ values of 4-ene-VPA and 2,4-diene-VPA were 0.01 ng/ml. R value for calibration curve were more than 0.999 in all compounds (Fig. 3). This method was applied to the phar-

Table II. *Recovery, Precision and Accuracy for VPA, 4-ene-VPA and 2,4-diene-VPA*

Concentration (μg/ml)	Recovery (%)	Precision (%)	Accuracy (%)
10	33.4	9.30	90.9
50	35.2	5.12	97.2
250	42.4	1.87	99.4

Valproic acid (VPA)

4-ene-VPA

Concentration (μg/ml)	Recovery (%)	Precision (%)	Accuracy (%)
0.02	72.2	14.2	113.2
0.1	79.4	3.24	104.3
0.5	70.1	2.16	94.2

2,4-diene-VPA

Concentration (μg/ml)	Recovery (%)	Precision (%)	Accuracy (%)
0.02	78.9	12.3	88.3
0.1	81.3	2.56	92.1
0.5	72.7	4.14	89.3

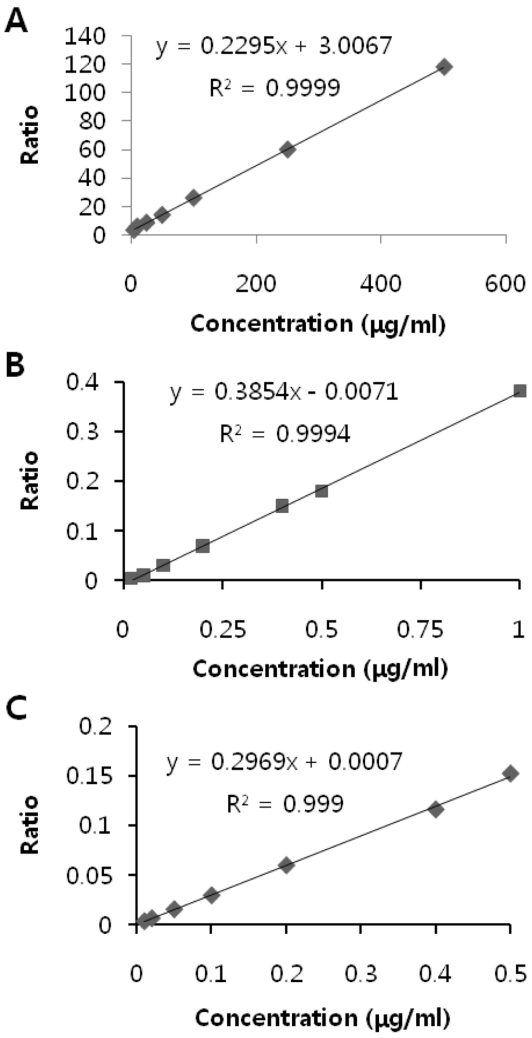

Figure 3. Calibration curves for valproic acid (VPA, A), 4-ene-VPA (B) and 2,4-diene-VPA (C).

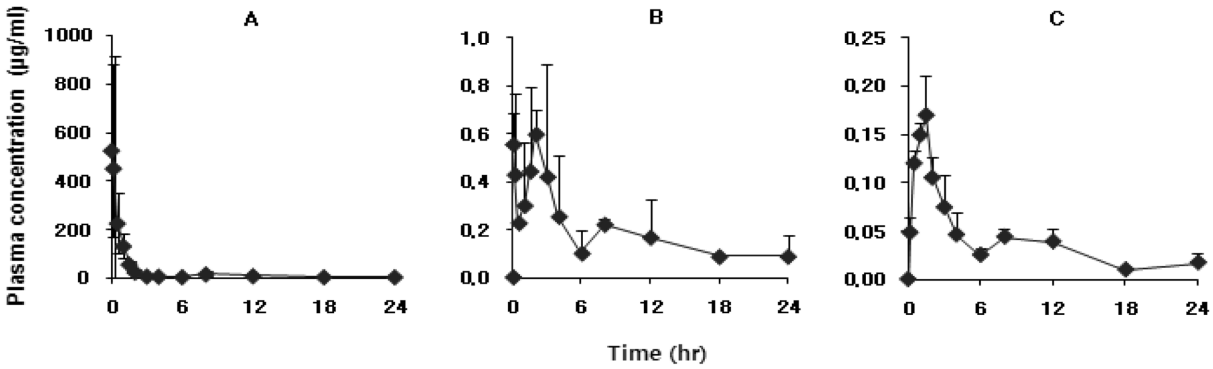

Figure 4. Plasma concentration–time profiles for valproic acid (VPA, A), 4-ene-VPA (B), 2,4-diene-VPA (C) after intravenous administration of VPA to rats at a dose of 100 mg/kg.

Table III–*Pharmacokinetic Parameters (mean±S.E) of Valproic Acid after Intravenous Administration at the Dose of 100 mg/kg.*

Parameters	Intravenous administration (100 mg/kg)
$AUC_{0\text{-infinity}}$ (μg·hr/mL)	341±98.3
MRT (hr)	2.84±1.30
Cl_t (mL/hr/kg)	0.90±0.02
$T_{1/2}$ (hr^{-1})	3.22±1.21
V_d (mL/kg)	2.56±1.10

macokinetic studied for VPA, 4-ene-VPA and 2,4-diene-VPA. Although large differences in the plasma level between VPA and its metabolites, we could successfully detect all plasma concentration both VPA and its metabolites simultaneously.

Pharmacokinetics of VPA, 4-ene-VPA and 2,4-diene-VPA

Temporal profile of VPA, 4-ene-VPA and 2,4-diene-VPA are shown at Fig. 4. The plasma profile of VPA following intravenous administration declined bi-exponentially as a function of time and calculated systemic clearance (Cl) and volume of distribution (V_d) were summarized at Table III.

After intravenous administration of VPA, C_{max} (maximum plasma concentration) for VPA ranged 500-800 μg/mL. On the other hand, C_{max} values of 4-ene-VPA and 2,4-diene-VPA were less than 1 μg/mL and reached after 2 hours from the drug injection. It means that very small portion of VPA is metabolized to toxic metabolites, but those low concentrations of toxic metabolites may be involved in the toxic effect in the liver. The maximum plasma concentration for 4-ene-VPA was higher by two fold than that of 2,4-ene-VPA. Therefore, 4-ene-VPA seems to be more related to the liver toxicity of valproic acid.

Conclusion

The sensitive method for the simultaneous determination of VPA, 4-ene-VPA and 2,4-diene-VPA in plasma was developed with gas chromatography and mass spectrometry. It was successfully applied to obtain plasma concentrations of VPA, 4-ene-VPA and 2,4-diene-VPA for the evaluation of pharmacokinetic and toxicokinetic profiles of VPA and its toxic metabolites.

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