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Original Article

Liquid chromatography–tandem mass spectrometry method for simultaneous determination of valproic acid and its ene-metabolites in epilepsy patient plasma ☆

Huan Lu [a], Chong Su [b], Lei Yin [b,c], Liqiang Gu [a], Jingkai Gu [b,c,**], Xiaohui Chen [a,*]

[a] School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, China
[b] Research Center for Drug Metabolism, College of Life Science, Jilin University, Qianjin Street, Changchun 130012, China
[c] Clinical Pharmacology Center, Research Institute of Translational Medicine, the First Hospital of Jilin University, Dongminzhu Street, Changchun 130061, China

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ABSTRACT

A simple and high throughput method was developed and validated for simultaneous determination of valproic acid and its two toxicant ene-metabolites, 2-enevalproic acid and 4-enevalproic acid in epilepsy patient plasma using liquid chromatography–tandem mass spectrometry. Probenecid was used as internal standard and solid-phase extraction was selected for sample preparation. A chromatographic separation was performed on an Agilent Poroshell SB-C_{18} column (50 mm × 4.6 mm i.d., 2.7 μm) by an optimized gradient elution at a flow rate of 0.9 mL/min. The total run time was 7 min. Electrospray ionization was used in negative ion mode by multiple reaction monitoring of the precursor-to-product ion transitions at *m/z* 143.0→143.0 for valproic acid, *m/z* 140.9→140.9 for 2-enevalproic acid and 4-enevalproic acid for their poor fragments, and *m/z* 283.9→239.9 for probenecid. The results showed good linearity of valproic acid, 2-enevalproic acid and 4-enevalproic acid in their respective linear ranges. The correlation coefficients were more than 0.998. The intra- and inter-day precision of the assay was less than 11.0% and the accuracy ranged from 2% to 12%. This analytical method was successfully applied to assay plasma concentrations of valproic acid and its two ene-metabolites in epilepsy patient plasma and used for therapeutic drug monitoring.

1. Introduction

Valproic acid (VPA, Fig. 1A) is one of the most widely used antiepileptic drugs, which has broad-spectrum antiepileptic activity.VPA possesses the best therapeutical effect on petit mal and shows individual difference among epilepsy patients [1]. It is notable that the administration of VPA may be closely associated with rare but serious hepatotoxicity with or without hepatonecrosis [2]. Metabolism is an uppermost route of VPA elimination, only with 1%–3% of an orally administered dose excreted unchanged by urine. The bioconversion of VPA relates to three important metabolic pathways: (i) approximately 50% of VPA is mediated by UDP-glucuronosyltransferases (UGTs); (ii) 40% of VPA experiences mitochondrial β-oxidation; (iii) almost 10% of VPA is metabolized by CYP3A4, CYP2C19 and CYP2C9 (cytochrome P-450) into oxidative metabolites. Glucuronidation and β–oxidation are the most important metabolic pathways of VPA [3]. 2-enevalproic acid (2-ene VPA) is provided by mitochondrial beta-oxidation of VPA and the formation of 4-enevalproic acid (4-ene VPA) is mediated by CYP2C9 [4]. Moreover, the structures of 2-ene VPA (Fig. 1B) and 4-ene VPA (Fig. 1C) are similar to those of hypoglycin and 4-pentenoic acid, known as hepatic toxicants [5–6]. VPA can be used alone or in combination with other antiepileptic drugs to treat epilepsy. Therefore, it is necessary to simultaneously monitor VPA and its toxicant, ene-metabolites in epilepsy patients. However, a small molecule with simple chemical structure is a challenge for the determination with tandem mass spectrometry. Especially, 2-ene VPA and its structural isomer 4-ene VPA both have the same precursor/product transitions, which is an analytical challenge to ensure their baseline separation.

Numerous techniques have been reported for the quantification of VPA in biological samples, mainly including fluorescence polarization immunoassay [7], thin layer chromatography-derivatization [8], homogeneous enzyme-linked immunoassay [9], gas chromatography [10], high performance liquid chromatography

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* Corresponding author.
** Corresponding author at: Research Center for Drug Metabolism, College of Life Science, Jilin University, Qianjin Street, Changchun 130012, China.
E-mail addresses: gujk@mail.jlu.edu.cn (J. Gu), cxh_syphu@hotmail.com (X. Chen).

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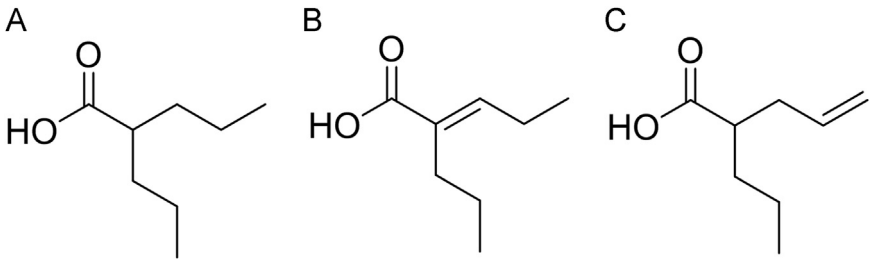

Fig. 1. Chemical structures of (A) VAP, (B) 2-ene VAP and (C) 4-ene VAP.

[11–16], and high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) [17]. However, the major disadvantages of these techniques were the low sensitivity and the long retention time. Gao et al. [18] used LC–MS/MS to simultaneously determinate VPA and 4-ene VPA. Because no stable ion fragments were produced during VPA, 2-ene VPA and 4-ene VPA ionization, the pseudo multiple reaction monitoring (pseudo-MRM) mode was chose to develop LC–MS/MS analytical method. However, Dziadosz et al. [19] adopted the components of the mobile phase with MRM as a novel way to analysis VPA in human serum with LC–MS/MS. In this study, we once tried to use the same method to determinate VPA, 2-ene VPA and 4-ene VPA, but this measure could not be applied to 2-ene VPA and 4-ene VPA. Because the concentrations of 2-ene VPA and 4-ene VPA are low, it is impossible to inspect signal when formic acid is added into mobile phase. Meanwhile, the concentration of VPA in pseudo-MRM mode is higher than that in MRM mode with aqueous 0.1% formic acid. Finally, pseudo-MRM was selected for determination of VPA, 2-ene VPA and 4-ene VPA. Wilimowska [20] quantified the variabity of concentrations of VPA and its selected metabolites, 2-ene VPA and 4-ene VPA. However, no specific LC–MS/MS method was reported for simultaneous determination of VPA and its two ene-metabolites, 2-ene VPA and 4-ene VPA, in biological specimens up to date.

In order to better explain pharmacodynamics and toxicokinetics of VPA, 2-ene VPA and 4-ene VPA in the body, we established a sensitive and high-throughput LC–MS/MS method for simultaneous determination of VPA, 2-ene VPA and 4-ene VPA based on solid-phase extraction (SPE) followed by gradient elution program in human plasma. Finally, the method was fully validated and successfully applied to therapeutic drug monitoring.

2. Materials and methods

2.1. Chemicals and reagents

VPA, 2-ene VPA, 4-ene VPA (purity $>98.0\%$) and probenecid (internal standard, IS, purity $>99.0\%$) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, USA). Formic acid (HPLC grade) was purchased from Tedia (Fairfield, OH, USA). Deionized water was obtained from a Millipore Milli-Q gradient water purification system (Molsheim, Franceo). Epilepsy patient plasma samples and heparinized blank (drug-free) human plasma samples were obtained from Changchun Blood Donor Service (Changchun, China).

2.2. Instruments

The LC–MS/MS system consists of an Agilent 1100 Series HPLC system (Agilent Technologies, Palo Alto, USA) and an Applied Biosystems Sciex Qtrap 5500 mass spectrometer (Applied Biosystems, Sciex, Ontario, Canada) equipped with an electrospray ionization (ESI) source. Acquisition and integration of data were achieved by Applied Biosystems Analyst Software, version 1.5.2.

2.3. LC–MS/MS condition

The separation of VPA, 2-ene VPA and 4-ene VPA was performed at 40 °C on an Agilent Poroshell SB-C_{18} column (50 mm × 4.6 mm i.d., 2.7 μm) protected by a SecurityGuard C_{18} guard column (4 mm × 3.0 mm i.d., Phenomenex Inc., USA) with a mobile phase that constituted water (A) and acetonitrile (B) at a flow rate of 0.9 mL/min. The initial mobile phase was composed of 10% B. After 4.0 min, the composition was changed to 60% B; these conditions were maintained for 0.5 min, after which the column was quickly equilibrated with the initial mobile phase.

MS parameters were optimized by infusing a standard solution of analytes or IS into the mass spectrometer through a syringe pump. The mass spectrometer with ESI source was operated in the negative ion mode. Nebulizer, heater and curtain gas pressure were set at 40, 40 and 30 psi, respectively; dwell time was optimized at 200 ms; ionspray voltage was regulated at −4500 V; heater gas temperature was maintained at 600 °C; declustering potentials (DP) −60 V and collision energies (CE) −12 eV were found to be the best for analytes and IS. Unit resolution was used for both Q1 and Q3 mass detection. MRM scan mode was used to monitor ion transitions at m/z 143.0→143.0 for VPA, m/z 140.9→140.9 for 2-ene VPA and 4-ene VPA, and m/z 283.9→239.9 for IS. Data were collected and processed by Applied Biosystems Analyst Software.

2.4. Preparation of calibration standards and quality control (QC) samples

The stock solutions of VPA, 2-ene VPA and 4-ene VPA (1 mg/mL) were successively prepared in ultra-pure water, stored in a refrigerator at 4 °C prior to use, and then mixed correspondingly to get mixed stock solution. Mixed working solutions of VPA, 2-ene VPA and 4-ene VPA were prepared by diluting prepared mixed stock solution to 20, 40, 60, 80, 100 and 125 μg/mL for VPA, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.25 μg/mL for 2-ene VPA, and 0.02, 0.04, 0.06, 0.08, 0.10 and 0.125 μg/mL for 4-ene VPA with drug-free human plasma. QC samples were prepared independently in the same way with concentrations of 30, 80 and 125 μg/mL for VPA, 1.5, 4.0 and 6.25 μg/mL for 2-ene VPA and 0.03, 0.08 and 0.125 μg/mL for 4-ene VPA. A stock solution of probenecid of 1 mg/mL was also prepared in ultra-pure water and diluted with drug-free human plasma subsequently to obtain an IS working solution of 0.05 μg/mL.

2.5. Sample preparation

Frozen human plasma samples from subjects were allowed to thaw in a water-bath at room temperature and vortex-mixed