A New Derivatization Method to Enhance Sensitivity for the Determination of Low Levels of Valproic Acid in Human Plasma

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A novel and sensitive high-performance liquid chromatographic (HPLC) method has been developed and validated for the determination of valproic acid (VPA) in human plasma. The method was based on derivatization of VPA using 2-bromo-2-acetonaphthone as a new derivatization reagent. Caprylic acid was used as an internal standard (IS). Under the optimized extraction and derivatization conditions, the method showed good linearity in the range of 0.05–200 μg mL⁻¹ and the limit of detection was as low as 0.01 μg mL⁻¹. The relative standard deviation for intra-day and inter-day (n = 5) was <5%. The recovery ranged from 95.2 to 101.4%. The proposed method is proved to be highly sensitive, simple and rapid, and was successfully applied to the analysis of VPA in plasma samples from patients with generalized epilepsy.

Introduction

Valproic acid (2-propylvaleric acid, VPA) is an effective antiepileptic drug in patients with several types of seizures, especially in those with idiopathic and symptomatic generalized epilepsies (1), generally regarded as the first choice agent (2). VPA shows dose-dependent neurological adverse reactions and large inter-individual pharmacokinetic variability (3); therefore, a simple, rapid and highly sensitive method for the determination of VPA in human plasma is very important for therapeutic drug monitoring (TDM) and to quantify VPA levels in bioequivalence and pharmacokinetic studies (4).

To date, several analytical approaches have been used in analysis of VPA levels such as gas chromatography (GC) (5, 6), capillary zone electrophoresis (7, 8), fluorescence polarization immunoassay (9), high-performance liquid chromatography (HPLC) (10–12), liquid chromatography-mass spectrometry (LC–MS) (13, 14) and LC–MS-MS (15, 16). Among them, HPLC method is commonly and widely applied due to its high separation efficiency and relatively low cost. VPA is a C₈-branched carboxylic acid and has no chromophore. Ultraviolet (UV) detection at 210 nm is non-specific due to the interference of biological matrix. Application of the HPLC method usually after derivatization with a suitable chromophore (18) is the preferred approach (19–20). Various chemical derivatization reagents including 2,4-dibromoacetophenone (21), 2-bromoacetophenone (22), 2-bromo-4-nitroacetophenone (23), α-bromoacetophenone (24) have been employed for the derivatization of VPA. These derivatization methods are simple; however, the sensitivity is not adequate in some routine TDM or pharmacokinetics studies (25–27). Further sensitivity is gained through fluorescent derivatization using 9-aminophenanthrene (28), 7-dimethoxycoumarin (BrMMC) (29), 2-(2-naphthoxy) ethyl-2-(piperidino)ethanesulfonate (NOEPES) (30), but those methods need long reaction time (1 h, 85°C) and the expensive derivatization reagent is hard to obtain.

Derivatization of VPA using NOEPES needs toluene, which is a toxic solvent. The objective of this study is to develop a simple, rapid and highly sensitive derivatization method for the determination of VPA in human plasma. To our knowledge, 2-bromo-2-acetonaphthone (BAN) has already been employed in the synthesis of fatty naphthacel esters (31, 32), the results of which indicated that the detection of naphthyl derivatives was shown to be highly sensitive (limit of detection, LOD, was found to be ~0.1 ng which was more than 10 times less than previous methods reported for fatty acid phenacyl esters). Milan et al. (33) derived ursodeoxycholic acid using BAN, the LOD was calculated to be 25 ng mL⁻¹. BAN can form naphthyl derivatives with compounds containing a free carboxylic acid group (34) and the resulting naphthyl derivatives have stronger UV absorbance than phenacyl esters, but there is no reference available on application of this derivatization reaction for analysis of VPA.

This work describes a new HPLC method after pre-column derivatization with BAN for the determination of VPA in human plasma. The reaction scheme is shown in Figure 1. A series of parameters affecting the derivatization–extraction efficiency were optimized and the comparison with previous derivatization method was also studied. Compared with existing derivatization methods, the advantages of this method were less time consuming and more sensitive with an LOD of 0.01 μg mL⁻¹ which could reach the same level of it by LC–MS. Furthermore, the presented method can provide a new strategy for the derivatization of other carboxylic acid drugs.

Experimental

Chemicals and Materials

Sodium valproate (No. 100963-201101) and sodium caprylate (IS) were obtained from the National Institute of Control of Pharmaceutical and Biological Products (Beijing, China). BAN (No.1104150) was purchased from Aladdin Chemistry Co. Ltd, 2-bromo-4-nitroacetophenone was obtained from Sigma-Aldrich. 18-Crown-6 ether (No. A1249) was provided by Aalfa Aesar—A Johnson Matthey Company (Ward Hill, MA). Blank plasma was provided by The Second Hospital of Hebei Medical University (Hebei, China). Methanol and acetonitrile (HPLC grade) was supplied from Kangside Scientific (Tianjin, China). Deionized water was prepared using the Milli-Q50 water purification System (Millipore, Bedford, MA, USA). All other chemicals and reagents were of analytical grade.
Apparatus and HPLC conditions
Analysis was performed on a HPLC system consisting of an L-6200A ternary pump (Hitachi, Japan) and a 785A UV detector (Applied Biosystems, USA). The data were collected by a HW-2000 chromatograph data workstation (Qianpu Corp., Nanjing, China) and the chromatograph was monitored at 251 nm. A Z-52 centrifuge from Baiyang (Shanghai, China), a XW-80 Vortex mixer (Shanghai medical university Instrument Co., Shanghai, China) and a QGC-12 T Nitrogen blowing instrument (Quandao Corp., Shanghai, China) were employed here. Separations were accomplished on a Diamonsil C18 column (150 mm/4.6 mm, 5 μm, Dikma, China) at room temperature. Elution was performed at a programmed flow-rate of 1 mL min⁻¹ with methanol/water at 80:20 (v/v).

Standard solution and quality control samples
The stock solution of VPA-sodium salt was prepared in acetonitrile at a concentration of 2 mg mL⁻¹. A series of working standards at appropriate concentrations were prepared by diluting stock solution with acetonitrile. A standard stock solution of sodium caprylate (IS) at 1 mg mL⁻¹ in acetonitrile was diluted to a working solution of 250 μg mL⁻¹. BAN (25 mg mL⁻¹) and 18-crown-6 ether (4 mg mL⁻¹) stock solutions were prepared in acetonitrile. All the stock solutions and working solutions were stored at −20°C and 4°C, respectively.

Appropriate aliquots of the above-mentioned working solutions of VPA-sodium salt and IS were added into human blank plasma so as to prepare quality control (QC) samples at three concentration levels of 0.05, 5.00, 100 μg mL⁻¹ for VPA-sodium salt and 25 μg mL⁻¹ for IS, respectively.

Sample collection and preparation procedure
Plasma samples were obtained from 12 epileptic patients aged from 8 months to 25 years old from the Second Hospital of Hebei Medical University participated in our study. They were diagnosed as epileptic and had received VPA as mono-therapy at 30 mg kg⁻¹ day⁻¹ twice daily for at least one month. The study protocol was approved by the Ethics Committee of the Second Hospital of Hebei Medical University, and each subject or their guardians gave written informed consent to participation. Approximately 2 mL of blood was collected in a centrifuge tube containing heparin at 12 h after the evening dose. After a 20 min centrifugation at 4.0 × 10³ g, the obtained plasmas for analysis were then stored immediately at −80°C until assayed according to the proposed method.

To 200 μL of the plasma sample, 200 μL of sulfuric acid (1 M) was added, the mixture was vortex-mixed for 30 s and extracted with 2 mL of n-hexane using a mechanical shaker for 5 min. After centrifugation at 1.25 × 10³ g for 5 min, 600 μL of supernatant was separated and 20 μL of potassium carbonate (0.01 M) was added, the mixture was evaporated to dryness under a gentle stream of nitrogen at 40°C after vortexing for 30 s.

Derivatization
The residue was dissolved with 50 μL of BAN (10 mg mL⁻¹) and 10 μL of 18-crown-6 ether (1 mg mL⁻¹). The mixture was vortexed for 30 s and derivatized in a water bath at 65°C for 20 min. Subsequently, 20 μL aliquot was introduced for HPLC analysis.

Results
Method validation
Specificity
The specificity of the method was evaluated by comparing the chromatogram of blank plasma, human blank plasma spiked with
IS, blank plasma spiked with VPA and IS and plasma sample from patients. Figure 2 shows the typical chromatograms for the analysis of VPA, indicating that there was no significant interference from endogenous compounds peak.

**Linearity, Limits of detection and quantification**

To 160 μL of blank plasma, standard working solutions (20 μL of VPA and IS, respectively) to reach nominal concentrations of 0, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 200 μg mL⁻¹ for VPA and a fixed concentration (25 μg mL⁻¹) of IS were added. The samples were analyzed as described above. The calibration curve was constructed by plotting the peak area ratio (VPA-BAN vs IS-BAN) as a function of the concentration of the spiked standard solutions using a 1/c² weighted linear least-squares regression model. The LOD and limit of quantification (LOQ) were determined separately in five replicates at signal-to-noise ratios (S/N) of 3 and 10, respectively. The linear range was 0.05–200 μg mL⁻¹ and the calibration equation was $R = 0.0314C - 0.0006$ with a correlation coefficient of 0.998 by using a weighted linear regression method. The determined LOD and LOQ were 0.01 and 0.05 μg mL⁻¹, respectively.

To justify the sensitivity of the present method, the linear range, LOD and LOQ for the determination of VPA in human plasma using present method was compared with other previous methods, whose results are summarized in Table I. Clearly, compared with those earlier derivatization methods, the presented method exhibits a wide range of linearity, very low LOD and LOQ. In addition, the LOD of this work is comparable to the LOD from GC-MS/MS method.

**Accuracy, precision and extraction recovery**

Experimental intra-day accuracy and precision were evaluated by analyzing five replicates at three concentration levels with the spiked QC samples. Inter-day accuracy and precision were calculated after repeated analysis in five different analytical run. Extraction recovery (ratio percentage) was calculated by comparing the peak area obtained from the QC samples after extraction and derivatization procedure to the peak areas obtained from the analysis of standard solutions without extraction.

Table II summarizes the intra-day, inter-day accuracy and precision. Extraction recovery for the VPA and IS from the QC samples is shown on Table III. All intra-day and inter-day accuracy

![Figure 2. Typical chromatograms of VPA and IS naphthacyl derivatives. (A) Blank plasma; (B) human blank plasma spiked with 25 μg mL⁻¹ of the IS; (C) human blank plasma spiked with 50 μg mL⁻¹ of VPA and 25 μg mL⁻¹ of the IS; (D) plasma sample collected from patient; 1 is the peak of VPA naphthacyl derivative, 2 is the peak of the IS naphthacyl derivative.](image)
Inter-day and intra-day Accuracy and Precision for Determination of VPA in Human Plasma (n = 5)

<table>
<thead>
<tr>
<th>Concentration added (µg mL⁻¹)</th>
<th>Concentration found (µg mL⁻¹)</th>
<th>Accuracy (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.0491</td>
<td>-1.8</td>
<td>4.3</td>
</tr>
<tr>
<td>5.00</td>
<td>5.07</td>
<td>0.7</td>
<td>2.7</td>
</tr>
<tr>
<td>100</td>
<td>100.2</td>
<td>0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.0491</td>
<td>-2.2</td>
<td>4.8</td>
</tr>
<tr>
<td>5.00</td>
<td>4.97</td>
<td>-0.6</td>
<td>4.0</td>
</tr>
<tr>
<td>100</td>
<td>100.7</td>
<td>0.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*Accuracy has been calculated as a percentage of the nominal concentration.

Table III

The Recovery of the Method for Determination of VPA and IS Concentration Spiked in Human Plasma (n = 5)

<table>
<thead>
<tr>
<th>Concentration added (µg mL⁻¹)</th>
<th>Concentration found (µg mL⁻¹)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
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<tr>
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<td>0.05</td>
<td>0.0495</td>
<td>99.0</td>
<td>1.6</td>
</tr>
<tr>
<td>0.049</td>
<td>97.8</td>
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</tr>
<tr>
<td>0.0493</td>
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<td>4.89</td>
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<td>5.07</td>
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</tr>
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<td>4.76</td>
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</tr>
<tr>
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<td>99.3</td>
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<td>1.0</td>
</tr>
<tr>
<td>98.8</td>
<td>98.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100.7</td>
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<td>IS</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>25.2</td>
<td>100.8</td>
<td>0.2</td>
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<td>24.7</td>
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<td>100.0</td>
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<tr>
<td>23.9</td>
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</tr>
<tr>
<td>24.8</td>
<td>99.2</td>
<td></td>
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</tr>
</tbody>
</table>

Stability

Six aliquots of QC samples at each of three concentration levels were analyzed to investigate the stability of samples. Long-term stability was assessed by analyzing the QC samples kept at the storage temperature (−20°C) for 4 weeks. Freeze–thaw stability was evaluated that samples were subjected to freezing for 24 h at −20°C and thawing at room temperature for three cycles. The stability of the derivative at room temperature after derivatization was also studied by analyzing the QC samples over a period of 24 h. The results in Table IV indicated that there is no stability related problems to be considered during the whole analysis.

Application of plasma samples in epileptic patients

The new developed method was successfully applied to determination of human plasma concentrations of VPA after oral administration. The results are shown in Table V. The therapeutic concentration of VPA ranges from 50 to 100 µg mL⁻¹. But our results indicated that the VPA plasma concentrations varied greatly among patients, in six cases the VPA concentrations were found to be below 50 µg mL⁻¹ and one was more than 100 µg mL⁻¹. So TDM of VPA is of great importance to avoid lack of efficacy at standard dosages or dose-related side effects.
Discussion

Optimization of extraction and derivatization conditions

The conditions of extraction and derivatization should be optimized in order to make the reaction complete and bring about a less consumption as well. The effects of the solvent selection, the amount of sulfuric acid, the reaction time, temperature and the concentration of BAN, 18-crown-6 ether were investigated. All parameters were optimized by using a 200 μg mL⁻¹ VPA QC samples as well as an excess derivatization reagent (25 mg mL⁻¹) and catalyst (4 mg mL⁻¹). All the contributing factors but one remain constant in every step of the optimization procedure, then the optimized value is used for the following experiments.

Solvent selection

It is essential to select a suitable organic solvent: five organic solvents including ether, ethyl acetate, diethyl ether, chloroform and n-hexane were used for the extraction of VPA, the results indicated that n-hexane provided best extraction efficacy in our study (Figure 3). So we chose n-hexane as the extraction solvent in the following experiments.

Amount of sulfuric acid

The pKₐ of VPA is 5, so VPA exists in a neutral form at a low pH. Because the acidic medium is required for the extraction of VPA, different amounts of sulfuric acid (1 M) in the range 100–300 μL were added to the sample solution to investigate the effect of amount of sulfuric acid on the extraction. The best amount for extraction was 200 μL in our experiment (Figure 4).

Reaction temperature, time and other parameters

We also evaluated different reaction times and reaction temperatures. An arbitrary time (20 min) was first set in order to test the peak area of VPA at different temperatures of 40, 50, 60, 65, 70, 80 and 100 °C. Figure 5 presents the effect of reaction temperature on the yield of VPA naphthacyl derivative, obtained by plotting the peak area as a function of temperature. As can be seen, the peak area increased when the temperatures were <60 °C, after which the peak area was unchanged. So, we choose 65 °C for subsequent experiments. Then we evaluated different reaction time (5, 10, 15, 20, 25, 30 and 60 min) with same samples and same derivatization reagent and catalyst. The peak area increased with the increase of reaction time from 5 to 15 min and then remained almost constant (as shown in Figure 6). Thus 20 min was used to subsequent experiments. The concentration of the derivatization reagent and the catalyst was also optimized using the same method and at last 2-bromo-2-acetonaphthone...
(10 mg mL\(^{-1}\)) and 18-crown-6 ether (1 mg mL\(^{-1}\)) were used in the following experiments (data not shown).

**Absorption spectrum of naphthacyl derivatives for VPA**

There are some reports describing the detection at 246 nm for naphthacyl derivatives of many fatty acids (31), at 254 nm for prostaglandin naphthacyl derivatives (32) and at 245 nm for ursodeoxycholic acid naphthacyl derivatives (33). The detection of the naphthacyl derivatives of VPA should be at its maximum absorbance wavelength in order to achieve higher analysis sensitivity. The absorbance spectrum of naphthacyl derivatives for VPA between 200 and 400 nm was shown in Figure 7. This spectrum displayed a maximum absorbance at 251 nm. Therefore,
Comparison of the Derivatization Efficiency of 2-bromo-2’-acetonaphthone and 2-bromoaacetophenone for Determination of VPA in Human Plasma*

<table>
<thead>
<tr>
<th>Derivatization reagent</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPA</td>
</tr>
<tr>
<td>2-Bromo-2’-acetonaphthone</td>
<td>$1.498 \times 10^4$</td>
</tr>
<tr>
<td>2-Bromoaacetophenone</td>
<td>$1.392 \times 10^5$</td>
</tr>
</tbody>
</table>

*Quality control samples: 50 μg mL$^{-1}$ for VPA and 25 μg mL$^{-1}$ for IS; extraction solvent: 2 mL n-hexane; derivatization: 50 μL of BAN (10 mg mL$^{-1}$) and 10 μL of 18-crown-6 ether (1 mg mL$^{-1}$) at 85°C for 20 min. Each value is the average peak area of five aliquots.

detection at 251 nm for naphthacyl derivatives of VPA was applied in this work.

**Comparison with previous method**

For a routine application, we compared our approach with the previous method where acetophenone was always used as a derivatization reagent to check whether our technique is indeed superior to theirs. For a complete evaluation of the two methods, five independent QCs (50 μg mL$^{-1}$ for VPA and 25 μg mL$^{-1}$ for IS) was used. Previous method differed from ours in that 2-bromoaacetophenone was used as the derivatization reagent, the remainder of the respective procedures was the same. The results in Table VI indicated that the peak area with our method has improved 10-fold in comparison with the 2-bromoaacetophenone derivatization.

**Conclusions**

In this study, a highly sensitive, simple and cost-effective method consisting of derivatization with BAN and HPLC was developed. Although numerous reagents have been used for determination of VPA, this is the first report in which BAN has been applied as a derivatization reagent to check whether our technique is indeed superior to theirs. For a complete evaluation of the two methods, five independent QCs were used. Previous method differed from ours in that 2-bromoaacetophenone was used as the derivatization reagent, the remainder of the respective procedures was the same. The results in Table VI indicated that the peak area with our method has improved 10-fold in comparison with the 2-bromoaacetophenone derivatization.

**Funding**

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