Determination of Valproic Acid without Derivatization in Human Plasma using High Performance Liquid Chromatography-Photodiode Array

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Abstract

Objective: To develop and validate a simple and sensitive HPLC method without derivatization for determination of valproic acid in human plasma. Methods: Nonanoic acid as internal standard was added to 500 µL of plasma sample prior to liquid-liquid extraction using n-hexane and 0.5% triethylamine. Chromatographic separation was achieved on C-8 Symmetry® column (5µm; 150 x 3.9mm) in isocratic mode at 45°C. The mobile phase was 40 mM sodium dihydrogen phosphate pH 3.5 – acetonitrile (56:44 %v/v) with flow rate of 1.00 mL/min and was detected at 210 nm. Method validation referred to EMA Guideline 2011 for bioanalytical method validation in term of parameters lower limit of quantification (LLOQ), selectivity, calibration curve and linearity, carry over, recovery and stability. The valid method was applied in pharmacokinetic study of one healthy subject after administration of 500mg extended release caplet of valproic acid. The blood was collected as much as 7mL for twelve spots, which are predose, 1, 2, 3, 4, 5, 8, 10, 24, 36, 48, and 72 hours after drug administration. Results: The calibration curve valproic acid was linear over the concentration range of 2.0 – 200.0 µg/mL (r = 0.9992). Within-run and between-run precision and accuracy were studied at four concentrations and RSDs were less than 1.8 % and 5.4 %, while the bias (accuracy values) were less than 17.9 % and 13.1 %, respectively. Conclusion: The developed method provides sensitivity, linearity, precision, accuracy and is suitable for analysis of valproic acid in plasma samples for pharmacokinetic studies.

Keywords: Valproic acid, Nonanoic acid, HPLC, Validation, Liquid-liquid extraction.

Introduction

Valproic acid (2-Propyl pentanoic acid) is a broad spectrum antiepileptic drug and is used in the treatment of primary generalized seizures, partial seizures and myoclonic seizures [1-3]. It has narrow therapeutic index with range concentration in plasma of 40-100mg/L (280 – 700 mol/L) [4]. Therefore therapeutic drug monitoring and bioequivalence study are needed to be done prior developing its generic drug. It requires sensitive and selective method so that it can measure the low drug level in plasma.

Many analytical methods have been developed for determination of valproic acid in biological matrices. Most chromatographic methods require long sample preparation and prior derivatization. Since valproic acid does not have chromophore group in its structure, most of the methods have been developed using HPLC with UV detection after derivatization with 2,4-dibromoacetophenone [5,6]. Previous research using chromatographic separations were carried out on C18 column with derivatization method. The fluorogenic agent used Br-MAMC with limit of quantification of 6µg/mL [7].Other methods have been developed for determination of valproic acid in human plasma using gas chromatography (GC). Several researches used dimethylsilyl and aminophenantrene as derivative agents in the determination of valproic acid [8,9]. This method is relatively more complex compared with HPLC because it also needs derivatization. Analysis of valproic acid
without prior derivatization will generate significantly efficient analysis time [10]. Most of the methods without prior derivatization had been developed in the previous researches using C18 or CN column for separating the analytes.

The aim of this study was to develop an accurate, efficient, selective, and sensitive HPLC-photodiode array method for determination of valproic acid and nonanoic acid as internal standard in human plasma without derivatization using C8 column and applying the method in an in vivo study. The liquid-liquid extraction procedure was used for the sample preparation using hexane and triethylamine.

Methods and Materials

Chemical and Reagents

Valproic acid was purchased from United States Pharmacopeia (USP), nonanoic acid as internal standard was purchased from Sigma-Aldrich, methanol High Performance Liquid Chromatography (HPLC) grade, acetonitrile HPLC grade, phosphoric acid 85% analytical grade, sodium dihydrogen phosphate, acetic acid glacial, ammonium acetate, formic acid, ammonium formate, hydrochloride acid, triethylamine, n-hexane, and ethyl acetate were purchased from Sigma-Aldrich, aquabidestilata (Ikapharmindo), human plasma with citrate anticoagulant was obtained from Indonesian Red Cross (IRC).

Chromatographic System

The assay was performed on Waters 2695 series HPLC system equipped with photodiode array detector (Waters2996). Separation was performed on a C-8 column (Waters, Symmetry® 5 µm; 150 x 3.9 mm), with a mobile phase of acetonitrile-40mM phosphate buffer (pH 3.5; 44: 56 v/v). The analyses were run at a flow rate of 1 mL/min, column temperature of 25 °C, and were detected by a photodiode array detector at 210 nm wavelength.

Calibration Standards and Quality Control Samples

A stock solution of valproic acid was prepared in mobile phase (40mM sodium dihydrogen phosphate buffer pH 3.5 – acetonitrile) at 1.0 mg/mL and was further diluted with the mobile phase to obtain the following concentrations of 200μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL, 10 μg/mL, 2μg/mL and Quality Control (QC) solutions at 6 μg/mL, 100 μg/mL and 150 μg/mL, respectively. All of the standard solutions were stored in refrigerator (≤8°C) until being used. The calibration curve standards and quality control samples were prepared by spiking them with valproic acid and internal standard working solutions. It is used for method validation.

Sample Preparation

A plasma sample (500 μL) was transferred to polypropylene tube, the 100 μL of internal standard working solution 1.0 mg/mL, 100 μL of 1mM phosphoric acid and 3 mL of n-hexane were added. The mixture was vortex mixed for 2 minutes and centrifuged at 3000 rpm for 10 minutes. Then 2 mL of organic phase was transferred to the new polypropylene tube and 250 μL of 0.5% triethylamine was added and vortexed for 2 minutes. Afterward the organic phase was discarded, and 40 μL water phase was injected into HPLC system.

Method Validation

Plasma samples were quantified using the ratio of the peak area of valproic acid to that of IS as the assay parameter. For the calibration standards, peak area ratios were plotted against analyte plasma concentrations. The acceptance criteria for calibration curve was a correlation coefficient (r) of 0.995 or better. Lower limit of quantification (LLOQ) was defined as the lowest concentration at which the precision was expressed by relative standard deviation that is lower than 20% and inaccuracy (bias) was expressed by relative difference of the measurement and true value is within 20%. The method specificity was evaluated by screening six lots of blank plasma. Accuracy and precision were assessed by determination of QC samples with five replicates for four concentration levels on the same day (intra-day/within-run) and five replicates for four concentrations on different day (inter-day/between-run). The acceptance criteria for intra-and inter-day precision was 15% or better, and the inaccuracy was within 15% or better, except for LLOQ was 20% or better. The stability of the analytes and IS in plasma were assessed by analyzing QC samples at two concentrations (low and high), respectively, in triplicate (n = 3), under different temperature and timing conditions.

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The results were compared with those for freshly prepared QC samples, and the percentage concentration deviation was calculated [11].

**Application of the Method to in vivo Study**

The *in vivo* study was conducted in one healthy subject to see the pharmacokinetic profile. The ethics committee approved the protocol (No:487/H2.F1/ETIK/2014) and the subject provided written consent. Blood samples were obtained following oral administration of sustained release caplet containing 500mg of valproic acid. Blood samples (7 ml) of the venous blood were collected at pre dose and until 72 hours after drug administration in the heparinized tubes. After blood separation, plasma was frozen at -20°C until analysis.

**Results**

**Sample Preparation and HPLC Analysis**

Liquid chromatography method development begins with optimizing the mobile phase composition and column type. The feasibility of several compositions of acetonitrile with different buffers including acetate buffer, formate buffer, and phosphate buffer were tested for complete chromatographic resolution of valproic acid and nonanoic acid. The suitability, selectivity, and robustness of the method were investigated with C18 and C8 columns. The C-8 Symmetry® column (5µm; 150 x 3.9mm) provided good selectivity, sensitivity and peak shape for valproic acid and nonanoic acid compare to C18 column which no peak was appeared.

The mobile phase consisting of 40 mM sodium dihydrogen phosphate buffer pH 3.5 - acetonitrile (56: 44% v/v) with flow rate of 1.00 mL/min, column temperature of 45°C was found to be suitable during chromatography optimization. The retention times for valproic acid and nonanoic acid were observed at 5.04 and 9.04 min, respectively.

**Method Validation**

**Selectivity/specificity**

Typical chromatograms of blank plasma and blank plasma spiked with valproic acid at LLOQ and IS were shown in figure 1. No significant interfering peak was observed around the valproic acid and IS retention times. The chromatograms of QCL, QCM, and QCH were shown in figure 2.

![Fig.1 Chromatograms of (A) blank plasma and (B) plasma sample at LLOQ concentration](image1)

![Fig. 2 Chromatograms of (A) QCL concentration, (B) QCM concentration, and (C) QCH concentration](image2)

**Limit of Quantification and Linearity**

The calibration curves showed a good linearity in the concentration range of 2-200 µg/mL with correlation coefficient (r> 0.99) and LLOQ for valproic acid was 2
µg/mL with RSD and bias were 1.89% and -16.84%. The other research [12] used C-18 column with protein precipitation and was analyzed using LC-MS-MS. The result was reported to have LLOQ of 2.0 µg/mL, while in other research [13,14] that used C-18 column with protein precipitation, the LLOQ was 1.00 µg/mL.

**Accuracy and Precision**

Intra-day precision and accuracy, inter-day precision and accuracy were calculated from data obtained during a three-day validation period. At each of the three days samples spiked with 4 different concentrations that are LLOQ, QCL, QCM and QCH (each concentration n=5) were analyzed. Accuracy was evaluated by calculating the difference between measured value and nominal values (% bias), precision was calculated based on the coefficient of variation (%CV).

The intra-day accuracy (bias to nominal value) ranged between 0.15 and 17.88% with a precision of 2.09-3.63%. The inter-day accuracy (bias to nominal value) ranged between -2.83 and 13.00% with a precision of 2.86-5.39%. Based on the data, the analytical method is accurate and precise thus fulfills the acceptance criteria of EMEA guideline. The detailed data are shown in table 1.

**Recovery and Carry-over**

The extraction recovery was calculated by comparing the response obtained after extraction of spiked quality control samples with known concentration (6, 100, 150 µg/mL, each concentration n=3) with the response of non-extracted solution of valproic acid. The mean extraction recovery of valproic acid was ranged between 99.31 ±5.73 and 109.34 ±4.02 with CV not more than 15%. The detailed data are shown in table 2. While the detailed data carry over are shown in table 3. Based on the results, the extraction recoveries were consistent over the entire concentration range. Spiked quality control containing the analyte in concentrations representing the upper range of calibration curve were extracted and analyzed (n=5). Immediately after injection of the high concentration quality control sample a blank matrix sample was injected. The analyte measured in the blank was required to be ≤20% of LLOQ and for internal standard ≤5%. The result showed that no carry-over was observed for valproic acid including the internal standard.

**Table 1: The intra-day accuracy-precision and inter-day accuracy-precision**

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Intra-day (n=5)</th>
<th>Inter-day (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean conc. (µg/mL)</td>
<td>CV(%)</td>
</tr>
<tr>
<td>2.00</td>
<td>2.36 ±0.04</td>
<td>1.75</td>
</tr>
<tr>
<td>6.00</td>
<td>6.17 ±0.14</td>
<td>2.22</td>
</tr>
<tr>
<td>100.00</td>
<td>100.15 ±3.63</td>
<td>3.63</td>
</tr>
<tr>
<td>150.00</td>
<td>153.61 ±3.21</td>
<td>2.09</td>
</tr>
</tbody>
</table>

**Table 2: Extraction recovery of valproic acid from human plasma**

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.00</td>
<td>109.34 ±4.02</td>
<td>3.67</td>
</tr>
<tr>
<td>100.00</td>
<td>99.31 ±5.73</td>
<td>5.77</td>
</tr>
<tr>
<td>150.00</td>
<td>105.02 ±3.25</td>
<td>3.10</td>
</tr>
</tbody>
</table>

**Table 3: Carry over of valproic acid and IS in plasma**

<table>
<thead>
<tr>
<th>Blank Sample</th>
<th>Carry over of Valproic Acid (%)</th>
<th>Carry over of IS(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank-1</td>
<td>11.51</td>
<td>0.00</td>
</tr>
<tr>
<td>Blank-2</td>
<td>4.51</td>
<td>0.00</td>
</tr>
<tr>
<td>Blank-3</td>
<td>10.13</td>
<td>0.00</td>
</tr>
<tr>
<td>Blank-4</td>
<td>4.60</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Dilution Integrity

Dilution integrity test was conducted to assess whether the dilution process during the bioanalytical process is accurate, precise and reliable or not. If the in vivo measurement of valproic acid levels exceeds the upper limit of 200.0mg/mL, it meets the concentration range of the calibration curve. Based on the result we can dilute the sample until ¼ higher concentration. The results of dilution integrity can be seen in Table 4.

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Dilution Factor</th>
<th>Intra-day (n=5)</th>
<th>Inter-day (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean conc. (µg/mL)</td>
<td>CV (%)</td>
<td>Bias (%)</td>
</tr>
<tr>
<td>50.00</td>
<td>½</td>
<td>136.09 ±5.28</td>
<td>3.88</td>
</tr>
<tr>
<td>75.00</td>
<td>¼</td>
<td>67.00 ±3.23</td>
<td>4.82</td>
</tr>
</tbody>
</table>

Stability

Stability test performed by analyzing QCL and QCH samples of each of the three replicates.

Table 5: Long term stability of valproic acid in plasma

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean conc.(µg/ml)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>Mean conc.(µg/ml)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Day</td>
<td>6.14 ±0.16</td>
<td>2.54</td>
<td>2.33</td>
<td>154.49 ±4.19</td>
<td>2.71</td>
<td>2.99</td>
</tr>
<tr>
<td>8 Days</td>
<td>6.00 ±0.08</td>
<td>1.28</td>
<td>0.00</td>
<td>165.48 ±3.96</td>
<td>2.39</td>
<td>10.32</td>
</tr>
<tr>
<td>16 Days</td>
<td>6.05 ±0.55</td>
<td>9.05</td>
<td>0.83</td>
<td>158.58 ±9.82</td>
<td>6.19</td>
<td>5.72</td>
</tr>
<tr>
<td>26 Days</td>
<td>6.09 ±0.23</td>
<td>3.81</td>
<td>1.5</td>
<td>162.33 ±5.12</td>
<td>3.16</td>
<td>8.22</td>
</tr>
</tbody>
</table>

Table 6: Freeze and thaw stability of valproic acid in plasma

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Mean conc.(µg/ml)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>Mean conc.(µg/ml)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 cycle</td>
<td>6.14 ±0.16</td>
<td>2.54</td>
<td>2.33</td>
<td>154.49 ±4.19</td>
<td>2.71</td>
<td>2.99</td>
</tr>
<tr>
<td>3 cycles</td>
<td>5.79 ±0.08</td>
<td>1.32</td>
<td>-3.50</td>
<td>154.87 ±2.87</td>
<td>1.86</td>
<td>3.25</td>
</tr>
</tbody>
</table>

Table 7: Post preparation(auto-sampler) stability of valproic acid in plasma

<table>
<thead>
<tr>
<th>Hour</th>
<th>Mean conc.(µg/ml)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>Mean conc.(µg/ml)</th>
<th>CV (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hour</td>
<td>6.14 ±0.16</td>
<td>2.54</td>
<td>2.33</td>
<td>154.49 ±4.19</td>
<td>2.71</td>
<td>2.99</td>
</tr>
<tr>
<td>24 hours</td>
<td>5.49 ±0.34</td>
<td>6.25</td>
<td>-8.50</td>
<td>148.90 ±8.37</td>
<td>5.62</td>
<td>-0.73</td>
</tr>
</tbody>
</table>

For long-term stability test, the samples were stored in freezer temperature of -20 °C over a period of 8, 16 and 26 days. The results obtained showed that valproic acid in plasma sample is stable to be stored in -20 °C for at least 26 days. Freeze thaw stability test was also performed. Valproic acid was stable in plasma after at least three cycles of freeze and thaw. The results of auto-sampler stability suggest that valproic acid was stable for at least 24 hours in auto-sampler.

Short-term stability test of valproic acid and internal standard standard stock solution were performed at room temperature for 24 hours. Long-term stability of standard stock solution stored at 4 °C over a period of 8, 16, and 26 days.

The results obtained for inaccuracy (bias) of short-term stock solution stability of valproic acid was -0.96% to + 1.89% and for nonanoic acid was 1.14% to 1.41%. This indicates that the stock solution of valproic...
acid and nonanoic acid remain stable at room temperature for at least 24 hours. While for long-term stability, bias of valproic acid stock solution was -0.85% to +1.76% and for nonanoic acid was -1.89% to +0.28% during 16 days at storage of 4°C, while in the storage of 26 days inaccuracy were ≥ 2.0% both for valproic acid and nonanoic acid. Thus the standard stock solution valproic acid and nonanoic acid can be used for 16 days.

Discussion

The aim of the present research was to develop an accurate, rapid, and sensitive bioanalytical method without derivatization including an efficient and reproducible sample preparation for determination of valproic acid and nonanoic acid as internal standard. In this research, sample preparation or sample extraction for analysis was carried out by two methods; protein precipitation and liquid-liquid extraction.

Based on the previous researches sample preparation of valproic acid in plasma generally uses liquid-liquid extraction. The advantage of this method compared with protein precipitation is its ability to clean up the sample from many impurities or interferences in plasma thus extend the column life. On the other hand, liquid-liquid extraction method uses more solvent for extraction as well as more complicated and time consuming.

So it needs to be compared with faster and more efficient extraction method such as protein precipitation. The measured parameter was the peak area and the interference around the retention times of analytes and internal standard, in which the highest peak area, less interference, and high extraction efficiency is the most optimum method. Based on the result, the optimum method for sample preparation of valproic acid without derivationisation in plasma is the liquid-liquid extraction method using phosphoric acid as the acid with n-hexane as organic solvent and extracted again with 0.5% trimethylamine.

Application of Validated HPLC Method

In order to verify the sensitivity and selectivity of the validated method and because there are at least 10 metabolites of valproic acid that have been identified, such as 3-oxovalproic acid, 2-propylglutaric acid, 3-hydroxyvalproic acid, 4-hydroxyvalproic acid, 5-hydroxyvalproic acid, valpro-1,4-lactone, 2-propil-2-pentenoic acid, 2-propyl-4-pentenoic acid, and 4-ene valproic acid. Therefore, it is necessary to apply the validated method to the in vivo study such as pharmacokinetic study of one healthy subject after administration of sustained release caplet containing 500 mg of valproic acid.

Based on the data, valproic acid concentration was acquired then was plotted into pharmacokinetic profile curves which can be seen in figure 4. The obtained Cmax was 65.75 ug / mL with tmax of 24 hours. The 5% Cmax value was 3.29 μg/mL that is higher than LLOQ, which was 2.00 μg/mL. These results show that the validated analysis method has been successfully applied. The chromatogram of plasma sample can be seen in figure 3.

Fig. 3 Chromatogram of plasma sample 24 h after oral dose of sustained release tablet containing 500 mg of valproic acid
Fig. 4 Pharmacokinetic profile curve of sustained release tablet that contains 500mg of valproic acid

Conclusion
In conclusion, the method that we developed using HPLC with UV detection without derivatization showed good sensitivity and selectivity with LLOQ of 2μg/mL using 500mL human plasma. The method was successfully applied in in vivo study for one healthy subject.

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Conflict of Interest
Declared none

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