Method Development and Validation of an HPLC Assay for the Detection of Hopantenic Acid in Human Plasma and Its Application to a Pharmacokinetic Study on Volunteers

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Summary. A reliable and sensitive reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet (UV) detection method was developed and validated for the quantification of hopantenic acid in human plasma. Hopantenic acid, with protocatechuic acid as the internal standard (IS), was extracted from plasma samples using a liquid–liquid extraction with methanol. A chromatographic separation was achieved on a Luna C18 column (4.6 mm × 150 mm, 5-μm particle size) and precolumn of the same sorbent (2.0 mm). An isocratic elution, at a flow rate of 1.0 mL min⁻¹, was used with a mobile phase consisting of acetonitrile, water, and 0.03% trifluoroacetic acid. The UV detector was set to 205 nm. The elution times for hopantenic acid and IS were ~4.3 and 5.4 min, respectively. The calibration curve of hopantenic acid was linear (r > 0.9994) over the range of 0.5–100 μg mL⁻¹ in human plasma. The limit of detection and limit of quantification for hopantenic acid were 0.034 and 0.103 μg mL⁻¹, respectively. The present method was successfully applied for the estimation of pharmacokinetic parameters of hopantenic acid following single oral administration of tablets containing 250 mg hopantenic acid to healthy volunteers. For hopantenic acid, the data showed a mean maximum plasma concentration (Cmax) of 2.32 μg mL⁻¹, with a time to reach peak plasma concentration (tmax) of 1.56 h.

Key Words: hopantenic acid, HPLC, human plasma, pharmacokinetic

Introduction

Hopantenic acid, (R)-4-[(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)amino]butanoic acid (calcium salt), has been used in the clinical praxis as a drug that acts on the central nervous system (CNS). Shown in Fig. 1, it is a natural homologue of pantothenic acid, in which the β-alanine is replaced on γ-aminobutyric acid (GABA). Hopantenic acid is a natural metabolite of GABA in nervous tissue. It was shown that when adding labeled GABA to brain homogenate and other tissues in vitro hopantenic acid was formed.
It has been shown that hopantenic acid can act directly with GABA receptors [1]. It was established that this compound is hardly metabolized and is excreted in the urine almost structurally intact [2]. Therefore, unlike GABA, the action of hopantenic acid can continue for a longer period of time, possibly resulting in greater action on the CNS.

Information on the methods of analysis for hopantenic acid is limited in the literature. There are only a few publications demonstrating the application of gas chromatography–mass spectrometry (MS) methods with multiple ion detection for the determination of hopantenic acid in natural products and biological samples, including plasma and urine [3–7]. Reversed-phase liquid chromatography (RP-HPLC)-MS with electrospray ionization [8], RP-HPLC with UV detection [9], and MS [10] detection have been used for the analysis of pantothenic acid, which is similar in structure to hopantenic acid. In some of these works, hopantenic acid was used as an internal standard (IS) for pantothenic acid analysis in plasma [8, 10]. No validated methods for the determination of hopantenic acid in human plasma have been reported.

In continuation of our studies on the pharmacokinetics of active substances in vivo [11, 12], the purpose of the present work was the development and validation of a simple, reliable, and sensitive RP-HPLC method with UV detection for the determination of hopantenic acid in human plasma and its application to pharmacokinetic studies.

**Experimental Procedures**

**Chemicals, Reagents, and Solutions**

A reference standard of hopantenic acid (purity >99%) was obtained from JSC MIR-PHARM (Obninsk, Russia). Protocatechuic acid, used as the IS, was purchased from Sigma-Aldrich (St. Louis, MO) (purity >99.5%). The deionized water was purified using a Milli-Q water system (Millipore Research and Development, Bedford, MA). Acetonitrile (HPLC grade) was from SPK Cryochrom (St. Petersburg, Russia). Trifluoroacetic acid was obtained from Riedel de Haen (Germany). All other reagents and solvents were of analytical grade and commercially available. Blank plasma was ob-
Sample Preparation

Frozen human plasma samples were thawed at ambient temperature. Plasma samples (1 mL) were spiked with both 10 μL of IS (protocatechuic acid, 0.5 mg mL\(^{-1}\)) and 3.0 mL of methanol. The samples were then vortex-mixed for 15 min at 1000 rpm (Vortex Genius 3, IKA, Germany). Next, the sample tubes were centrifuged for 10 min at 1000 g (EBA21 table centrifuge, Hettich, Germany). The upper phase was transferred to a 5-mL polypropylene tube and evaporated to dryness under a stream of nitrogen at 45°C (N-EVAP 11155, Organomation, USA). The residue was dissolved in 0.5 mL of water containing 0.2% HCl, and 20 μL was then injected directly into the RP-HPLC system.

Chromatography

HPLC analyses were performed at room temperature using a Shimadzu HPLC system (Kyoto, Japan) consisting of two LC20AD pumps, a DGU-20 A3 degasser and a SPD-M20 A diode array detector. Separation was achieved using a Luna C18 (4.6 i.d. × 150 mm, 5 μm) column (Phenomenex, USA) and a SecurityGuard precolumn (2.0 mm) of the same sorbent (Phenomenex, USA). Data analysis was performed using LC Solution PC software (Shimadzu, Kyoto, Japan).

An isocratic elution, at a flow rate of 1.0 mL min\(^{-1}\), was used with a mobile phase consisting of acetonitrile, water, and 0.03% trifluoroacetic acid. UV detection was performed at room temperature, using a wavelength of 205 nm.

Method Validation

For the quantification of hopantenic acid, chromatograms of blank, spiked, and drug-administered volunteer plasma samples were compared to determine the level of interference by endogenous, co-eluting components.

Standard stock solutions of hopantenic acid (1.0 mg mL\(^{-1}\)) and IS (0.5 mg mL\(^{-1}\)) were prepared separately in methanol. These solutions were stored at 4°C and away from light and were found to be stable for at least 1 month. For the calibration curve, 10 different standards of hopantenic acid solution were prepared by the dilution of the stock solution. For each of the standard solutions, 1.0 mL was evaporated to dryness at 45°C under a stream of nitrogen gas. Then 1.0 mL of the drug-free plasma and 10 μL of
the IS solution were added. The calibration range was 0.5–100.0 μg hopan-
tenic acid per milliliter plasma. Calibration standards were extracted and assayed as described earlier. The calibration curve was constructed based on the peak area ratio of hopanenic acid to IS ($x$) versus the concentration of spiked hopanenic acid ($y$).

The accuracy and precision of the method were assessed by assaying five replicate quality control (QC) samples. The QC samples were prepared at three different concentrations in the same way as the calibration samples. To evaluate the repeatability (intraday precision) and the intermediate precision (interday precision), the same QC samples, consisting of three different hopanenic acid concentrations, were injected into the HPLC system 6 times on the same day and 6 times over different days, respectively. This allowed the percent relative standard deviations (% RSD) of the data to be calculated.

The extraction recoveries were determined by comparing the peak areas of hopanenic acid, obtained from the QC samples (5.0, 25.0, and 100.0 μg mL$^{-1}$, $n = 6$), to the IS at a concentration of 5.0 μg mL$^{-1}$ ($n = 3$). The recovery of hopanenic acid by methanol extraction was determined by comparing the observed peak area ratios in extracted biosamples to those of nonprocessed standard solutions. The first series of blank plasma samples, spiked with three different concentrations of hopanenic acid and protocatechuic acid, were extracted as described in the Sample Preparation section. The second series of blank plasma samples were spiked only with the IS. Three different concentrations of hopanenic acid were then added to the dry residues obtained after the methanol extraction.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations: LOD = $3\text{SD}/b$ and LOQ = $10\text{SD}/b$ [13, 14], where SD is the standard deviation, and $b$ is the slope of the calibration curve, with defined precision and accuracy under the given experimental conditions.

The stability of hopanenic acid in the plasma was assessed using QC samples on the three levels of concentration (5, 10, and 25 μg mL$^{-1}$) under three conditions: room temperature for 24 h, 37°C for 12 h, and −20°C for 14 days. Stability was expressed as a percentage of the nominal concentra-

Clinical Pharmacokinetic Study

This study was an oral single-dose, open, uncontrolled pharmacokinetic trial with a 14-day predrug washout period of 18 healthy, human volunteers. The study was carried out at the Clinical Pharmacological Unit (CPU) in the Department of Pharmacology at the St. Petersburg State Medical
Healthy men and women volunteers between 19 and 24 years of age, free from significant physical or psychiatric disorders as determined by medical history, physical examination, clinical chemistry, hematology, and urine analysis, were included in the study. Volunteers were excluded from the study if they had any significant abnormal test results or had received any drug treatment during the 3 months prior to the study. Volunteers gave written, witnessed, and informed consent in vernacular language prior to participating and were free to withdraw from the study at any time. The well-being of the volunteers was monitored throughout the study and 1 week after the withdrawal of the last blood sample. Any adverse events that occurred were recorded. Oral temperature, pulse rate, and blood pressure were monitored before drug administration and at intervals throughout the postdosing period. Adequate arrangements for entertainment were made for the volunteers so that they felt relaxed during the trial. Standard diet was supplied and included breakfast, lunch, snacks, tea, and dinner. Water was allowed *ad libitum*.

Participants were monitored for any adverse reactions, especially epigastric pain, nausea, vomiting, and diarrhea.

On the day of the trial, volunteers reported to the CPU at 09.00 h. After briefing, documentation, and acclimatization, a standardized breakfast was provided. Then an indwelling venous catheter (Vasofix no. 20) was fixed in the volunteer’s forearm using all aseptic precautions. Under supervision, one tablet containing 250 mg of hopantenic acid (Pantocalcin®; Valenta Pharmaceuticals, Schelkovo, Russia) was then administered orally with 200 mL of water. Volunteers remained inside the CPU until the last blood sample was taken, and during this time, they all received the same meals. Concomitant medication, tobacco, or other intoxicants were not permitted during the study. Venous blood samples (5 mL each) were drawn from each volunteer prior to drug administration and at 0.5, 1, 2, 6, 10, 24, 34, and 48 h after drug administration.

Blood samples were collected in heparinized Vacutainer™ tubes and were centrifuged (2000 rpm for 20 min) immediately to separate the plasma. The collected plasma was refrigerated until analysis and stored at –20°C prior to analysis. Specimens were thawed and allowed to reach room temperature before analysis. The concentration of hopantenic acid in plasma was then determined by HPLC.

Noncompartmental pharmacokinetic analysis of concentration–time data was performed [17]. The actual blood collection times were used for all
calculations. The area under the whole plasma concentration-time curve (AUC_{0-\infty}) was calculated using the trapezoidal rule. The maximum plasma concentration (C_{max}) and the time to reach the peak plasma concentration (t_{max}) were calculated directly from the experimental data.

The elimination rate constant (K_{el}) and elimination half life (t_{1/2}) were determined by a log-linear regression on the data from the terminal mono-exponential phase of the concentration versus time profiles.

Assuming complete absorption, clearance (Cl) and apparent volume of distribution (V_d) were calculated as Cl (mL min^{-1}) = K_{el} \times V_d/60 and V_d (mL) = dose/K_{el} \times AUC_{0-\infty}, respectively.

Values were reported as the mean ± SD, and the data were considered statistically significant at p < 0.05.

Results and Discussion

Method Development

The mobile phase composition is a critical factor for the separation of hopantenic acid from IS, protocatechuic acid, and any endogenous substances. Experimentally determined operational conditions (i.e. composition of the mobile phase, flow rate, and the method of elution) were chosen to provide optimal separation and measurement of hopantenic acid for subsequent routine analyses. Due to the absence of a specific maximum in the UV spectrum of a solution of hopantenic acid, a short wavelength range (200–210 nm), described as intensive but for nonspecific absorption, must be used. The detection wavelength was chosen to be 205 nm. Similar data are found in the literature for the analysis of pantothenic acid, which is a related compound [9, 18, 19].

In search of an appropriate and a simple mobile phase, several solvent mixtures containing acetonitrile or methanol and water were examined. Poor resolution was observed between hopantenic acid and protocatechuic acid with methanol in the mobile phase; therefore, acetonitrile was employed as the organic modifier. The mutual separation of hopantenic acid, protocatechuic acid, and endogenous substances was optimized using an isocratic elution with a mobile phase consisting of an acetonitrile-water mixture. The mobile phase pH was adjusted to 2.8–3.0 by use of an acid modifier, trifluoroacetic acid. The identity of hopantenic acid in the sample solution was confirmed (1) by comparing retention times and spectral parameters of the main component with those of the reference standard and (2) by adding the reference substance to the analyzed sample.

Banno et al. [5] directly purified the plasma samples, without deproteinization, on an ion-exchange resin, and the eluate was extracted with
ethyl acetate under acidic conditions. In other work, acidified plasma was deproteinized through extraction with chloroform, and the aqueous layer was subsequently extracted with ethyl acetate [3]. A number of solvents were examined to determine the best method for extracting hopantenic acid from human plasma samples. These solvents included ethyl acetate, chloroform, acetonitrile, methanol, acetone, and ethanol. Methanol gave a high extraction recovery for hopantenic acid from plasma samples without any significant interference from endogenous substances. Ethanol, ethyl acetate, and acetonitrile dissolved many endogenous co-eluents, resulting in a high back-pressure and short column life. Therefore, methanol was chosen as the most suitable extraction solvent.

Different compounds were tested as a potential IS to improve the precision of the RP-HPLC quantification. An IS is important for assaying biological samples. Of those tested, protocatechuic acid was selected as the most appropriate for the present analysis because it is stable and does not exist endogenously in plasma. Moreover, in the present study, protocatechuic acid did not interfere with the plasma sample matrix, and it was well separated from hopantenic acid.

**Method Validation**

The method was validated according to the International Conference on Harmonization guidelines for the validation of analytical methods [13, 14].

The representative chromatograms of blank plasma, spiked plasma standard, and real plasma sample are shown in Fig. 2. There are no co-

![Fig. 2. Representative chromatograms of: (a) a blank human plasma sample, (b) a blank human plasma sample spiked with hopantenic acid (10 μg mL⁻¹) or IS (5 μg mL⁻¹), and (c) a plasma sample from a volunteer 2 h after an oral administration of a 250-mg hopantenic acid tablet (1: hopantenic acid and 2: IS)]
eluting interference peaks from endogenous compounds near the hopan-
tenic acid and IS peaks. The retention times of hopanentic acid and the IS were about 4.3 and 5.4 min, respectively.

The calibration curve for hopanentic acid was linear over the concen-
tration range between 0.5 and 100 μg mL⁻¹. The regression equation was \( y = 0.02003x + 0.00846 \), where \( y \) is the hopanentic acid concentration in microgram per milliliter, and \( x \) is the peak area ratios of hopanentic acid to IS at 205 nm (\( r = 0.9994, \text{RSD} = 2.25\% \)). The LOD and the LOQ for hopanentic acid were 0.034 and 0.103 μg mL⁻¹, respectively. These results demonstrate good linearity, indicating that this method could be applied to plasma samples containing both low and high hopanentic acid levels.

The precision and accuracy of the method were examined by adding known amounts of hopanentic acid to the blank human plasma. For intra-
day precision and accuracy, six replicate QC samples at each concentration
were assayed on the same day. The interday precision and accuracy were evaluated over different days. The results are summarized in Table I. The intraday and interday precisions were within 5% for hopantenic acid, indicating that the method was reproducible.

**Table I.** Precision and accuracy for the determination of hopantenic acid in human plasma

<table>
<thead>
<tr>
<th>Concentration added (μg mL(^{-1}))</th>
<th>Intraday variability</th>
<th>Interday variability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration found (mean ± SD) (μg mL(^{-1}))</td>
<td>Precision (% RSD)</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>--------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>5.0</td>
<td>5.23 ± 0.12</td>
<td>4.7</td>
</tr>
<tr>
<td>25.0</td>
<td>24.3 ± 1.15</td>
<td>2.3</td>
</tr>
<tr>
<td>100.0</td>
<td>99.2 ± 3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Intraday: \(n = 6\); interday: \(n = 6\) series per day for 5 days

The mean extraction recoveries of hopantenic acid from spiked human plasma using the liquid extraction conditions were 96.0 ± 3.5, 98.9 ± 2.8, 95.7 ± 2.4% \((n = 3)\) at concentrations of 5.0, 10.0, and 25.0 μg mL\(^{-1}\), respectively. The extraction recovery of IS from human plasma was 95.4 ± 3.1% at a plasma concentration of 5.0 μg mL\(^{-1}\) \((n = 3)\). Therefore, the precision, accuracy, and recovery criteria for analyzing biological samples were achieved with the analytical method developed.

The stability of hopantenic acid in plasma (5.0, 10.0, and 25.0 μg mL\(^{-1}\)) at different temperatures is shown in Table II. A decrease of \(~12\%\) was observed in plasma samples after 24 h at room temperature. After 24 h at 37°C, \(~34\%\) of the hopantenic acid was degraded. However, the frozen samples, stored at −20°C for 14 days or treated after three freeze–thaw cycles, were stable.

**Table II.** Stability of hopantenic acid in human plasma at three QC levels \((n = 5)\)

<table>
<thead>
<tr>
<th>Nominal concentration (μg mL(^{-1}))</th>
<th>Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Room temperature (24 h)</td>
</tr>
<tr>
<td>5.0</td>
<td>89.4 ± 6.8</td>
</tr>
<tr>
<td>10.0</td>
<td>89.8 ± 4.3</td>
</tr>
<tr>
<td>25.0</td>
<td>85.6 ± 7.2</td>
</tr>
</tbody>
</table>
Clinical Pharmacokinetic Study

All the 18 enrolled volunteers completed the study. Subjects included 5 men and 13 women who had a mean age of 20.67 ± 0.41 years (range 19–24 years). Their mean height was 171.5 ± 1.6 cm (range 163–178 cm), and their mean weight was 64.72 ± 2.06 kg (range 50–83 kg).

When the plasma concentration of hopantenic acid versus time curve was plotted, an initial steep rise in plasma concentration was followed by a persistent fall (Fig. 3).

The hopantenic acid peak was seen in the plasma at 1.56 ± 0.12 h ($t_{\text{max}}$) (mean ± SD) after the administration of the drug. At this time, the plasma concentration was 2.3 ± 0.2 $\mu$g mL$^{-1}$ ($C_{\text{max}}$). The plasma curve declined with an elimination rate constant ($K_{\text{el}}$), averaging 0.11 ± 0.01 with a corresponding elimination half life ($t_{1/2}$) of 6.68 ± 0.25 h. The AUC$\text{0–48}$ average was 13.5 ± 0.7 $\mu$g h mL$^{-1}$ (Table III). Hopantenic acid was not detected in any of the volunteer’s plasma 48 h after drug administration.

![Fig. 3. The mean plasma concentration–time profile of hopantenic acid after oral administration of a 250-mg hopantenic acid tablet to healthy volunteers (mean ± SD)](image)

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Mean</th>
<th>SD</th>
</tr>
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<tbody>
<tr>
<td>AUC$\text{0–48}$ ($\mu$g h mL$^{-1}$)</td>
<td>13.5</td>
<td>0.7</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>9.64</td>
<td>0.36</td>
</tr>
<tr>
<td>$K_{\text{el}}$ (h$^{-1}$)</td>
<td>0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>6.68</td>
<td>0.25</td>
</tr>
<tr>
<td>$C_{\text{max}}$ ($\mu$g mL$^{-1}$)</td>
<td>2.3</td>
<td>0.2</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (h)</td>
<td>1.56</td>
<td>0.12</td>
</tr>
<tr>
<td>$C_{\text{max}}$/AUC$\text{r}$ (mL h$^{-1}$)</td>
<td>0.17</td>
<td>0.01</td>
</tr>
<tr>
<td>$V_{\text{d}}$ (mL)</td>
<td>3136</td>
<td>229</td>
</tr>
<tr>
<td>Cl (mL min$^{-1}$)</td>
<td>5.38</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Hopantenic acid was well tolerated by all volunteers as a single dose, orally administered 250 mg tablet formulation. No adverse events were observed or reported during the trial or throughout the following week.

This study was planned for the analysis of healthy volunteers using a single oral dose of 250 mg hopantenic acid. Although pharmacokinetic data for hopantenic acid were discussed in two research papers [6, 20], these data were not found despite an intensive internet search. We have reason to believe that this is the first published report on the pharmacokinetics of hopantenic acid in humans. Because there are no other published studies on the pharmacokinetics of hopantenic acid, a comparison is not possible.

**Conclusions**

For the first time, a reliable, simple, and sensitive isocratic RP-HPLC method with UV detection for the determination of hopantenic acid in human plasma was developed and validated. The method consisted of liquid-liquid extraction, centrifugation, and direct injection into the HPLC system. This method has been successfully applied to a pharmacokinetic study of hopantenic acid tablet formulation after an oral administration to healthy volunteers.

**Acknowledgment**

The authors are grateful to Valenta Pharmaceuticals (Schelkovo, Moscow region, Russia) for financial support.

**References**


Accepted by DA