A Simple and Rapid High-Performance Liquid Chromatography Tandem Mass Spectrometry Method for the Determination of Propafenone (PPF) and Its Active Metabolite 5-Hydroxypropafenone (5-OHP) in Human Plasma and Its Application in a Pharmacokinetic Study

Zhiyan Chi1,2, Ruijuan Liu1, Ya Li1,2, Keli Wang1,2, Chang Shu1, and Li Ding1,2,*

1Department of Pharmaceutical Analysis, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China and 2Nanjing Clinical Tech. Laboratories Inc., 18 Zhilan Road, Jiangning District, Nanjing 210009, China

*Author to whom correspondence should be addressed. Email: dinglidl@hotmail.com

Received 14 June 2016; Revised 26 March 2017; Editorial Decision 26 April 2017

Abstract

A simple and rapid high-performance liquid chromatography tandem mass spectrometry method for the determination of propafenone (PPF) and its active metabolite 5-hydroxypropafenone (5-OHP) in human plasma was developed and validated. This new method was linear and allowed simultaneous quantification of PPF and 5-OHP at a lower level of 0.5 ng/mL. The aliquot of 200 μL plasma sample was simply treated with 4-fold methanol to deproteinize the plasma. The chromatographic separation was achieved on a Hedera ODS-2C18 analytical column with the mobile phase of methanol and 5 mM ammonium acetate solution containing 0.2% formic acid (pH 3.2) (68:32, v/v) at a flow rate of 0.4 mL/min. Quantitation of the analytes was achieved by multiple reaction monitoring under positive ionization mode. The method was successfully applied to a pharmacokinetic study of PPF and 5-OHP in healthy Chinese volunteers. After oral administration of a single dose of 425 mg PPF hydrochloride sustained-release capsule, the maximum peak plasma concentration (Cmax) of PPF was 210.9 ± 141.9 ng/mL with a Tmax of 6 ± 1 h, the Cmax of 5-OHP was 129.6 ± 65.4 ng/mL with a Tmax of 7 ± 2 h. The area under plasma concentration–time curve (AUC0–36) of PPF was 1610 ± 1309 ng·h/mL with a t1/2 of 4.6 ± 1.1 h, the AUC0–36 of 5-OHP was 1446 ± 754 ng·h/mL with a t1/2 of 7.6 ± 1.6 h.

Introduction

Atrial fibrillation (AF) is one of the most common arrhythmias, which can cause symptoms such as palpitation, presyncope, chest tightness and dyspnea. Over time, AF can cause more serious complications, for instance, stroke and thromboembolism (1, 2). Propafenone (PPF) is a potent and generally well-tolerated antiarrhythmic agent which has been shown to be effective against a variety of cardiac arrhythmias, particularly AF (3, 4). As a class IC antiarrhythmic drug, PPF acts on the sodium and HERG ion channels and has weak β-blocking effect. Its active metabolite, 5-hydroxypropafenone (5-OHP), also has the antiarrhythmic activity (5, 6).

Because the instant-release formulation of PPF may result in fluctuations of drug concentrations in plasma that favor the occurrence of unwanted effects, a sustained-release formulation has been developed by more and more pharmaceutical companies to minimize fluctuations of drug concentrations in plasma and reduce the unwanted adverse events (7). As we all know, the sustained-release
formulation can delay the release of drugs, reduce the frequency of dosing and improve the compliance of patients. There are no studies focused on the pharmacokinetics of PPF and 5-OHP after oral administration of a single dose of PPF hydrochloride sustained-release capsule (PPF-SR) in Chinese healthy subjects. It is essential to develop a simultaneous quantification method for the two analytes to evaluate the PPF-SR and investigate the pharmacokinetics of PPF and 5-OHP in healthy volunteers.

Most reported HPLC–UV methods for the determination of PPF or 5-OHP had the very long run time, or were not sensitive enough for pharmacokinetic study of PPF or 5-OHP in human (8–15). Some reported LC–MS or liquid chromatography tandem mass spectrometry (LC–MS-MS) methods did not allow the simultaneous determination of PPF and 5-OHP in human plasma (16, 17). It is more difficult to quantify PPF and 5-OHP at the same time than only quantify PPF. Moreover, 5-OHP is an active metabolite of PPF in vivo and it is necessary to evaluate the pharmacokinetics of 5-OHP in human. The lower limit of quantification (LLOQ) of those LC–MS or LC–MS-MS methods developed for the simultaneous determination of PPF and 5-OHP could not meet the requirement (an LLOQ of 0.5 ng/mL) of our clinical pharmacokinetic research (18–20). Among the published LC–MS-MS methods, the method developed by Sujan Kumar et al. (21) was the only one meets the required sensitivity for our pharmacokinetic study of the PPF-SR in healthy volunteers, but the proposed Hybrid-SPE-Precipitation technique for sample preparation was tedious and time-consuming. Our LC–MS-MS method with simple pretreatment procedure and high sensitivity could also meet the requirement of the pharmacokinetic study of the PPF-SR. It was a highly sensitive, highly selective and high throughput method developed for the simultaneous quantification of PPF and 5-OHP in human plasma. The method can be applied to determine the concentrations of PPF and 5-OHP in human plasma and evaluate the pharmacokinetic properties of the PPF-SR in healthy volunteers.

Experimental

Chemicals and reagents

The reference standards of PPF hydrochloride (purity 99.9%), 5-hydroxypropafenone (5-OHP) hydrochloride (purity 99.5%) and quetiapine (internal standard, IS, purity 100.0%) were purchased from the National Institutes for Food and Drug Control (Beijing, China). The PPF hydrochloride sustained-release capsules (PPF-SR, 425 mg) were supplied by GlaxoSmithKline plc (GSK) Pharmaceutical Company (Brentford, London). Methanol was HPLC grade purchased from Merck KGaA (Darmstadt, Germany). Formic acid and ammonium acetate were analytical grade purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). Deionized water was produced by a Milli-Q Reagent Water System (Millipore, Bedford, MA, USA).

LC–MS-MS instrumentation and analytical conditions

The LC was performed on an Agilent 1200 Series LC (Agilent Technologies, Palo Alto, CA, USA), which equipped with an Agilent 1200 binary pump (model G1312B), vacuum degasser (model G1322A), Agilent 1200 autosampler (model G1367C), and temperature controlled column compartment (model G1330B). The chromatographic separation was carried on a Hedaera ODS-2C18 analytical column (150 × 2.1 mm², 5 μm; Hanbon Science and Technology, Hua’nan, China) with a security Guard-C18 column (4 mm × 2.0 mm, 5 μm; Phenomenex, Torrance, CA, USA). The isocratic mobile phase consisted of methanol and 5 mM ammonium acetate solution containing 0.2% formic acid (pH 3.2) (68:32, v/v) was delivered at a flow rate of 0.4 mL/min into the mass spectrometer. Autosampler and column temperature were set at 4°C and 38°C, respectively. The injection volume was 5 μL.

The mass detection was achieved by using an Agilent 6410B triple quadrupole mass spectrometer (USA) equipped with an ESI source (model G1956B). The mass spectrometer was operated in the positive ESI mode with the drying gas temperature of 350°C, drying gas flow rate of 12 L/min, nebulizer pressure of 50 psi and capillary voltage of 4,000 V. Quantification was achieved by multiple reaction monitoring (MRM) of the transitions of m/z 342.2 → 116.1, m/z 358.2 → 116.2 and m/z 384.2 → 253.1 for PPF, 5-OHP and IS, respectively. The fragmentor voltage values set for PPF, 5-OHP and IS were 127, 135 and 135 V, respectively. The collision energy set for PPF, 5-OHP and IS were 20, 22 and 20 eV, respectively. The system control and data analysis were performed by MassHunter Qualitative Analysis Software (B.03.01 Build 346).

Stock and standard solutions

The mixed stock solution containing accurately weighed reference compounds was directly prepared in methanol. The mixed working standard solutions were prepared by diluting the mixed stock solution with 50% aqueous methanol (v/v) to obtain a series of concentrations for the calibration curve. As for the IS, it was prepared by diluting the stock solution with methanol to get the working solution with the concentration of 1.087 μg/mL. All the solutions were stored at ~20°C until use.

Calibration and quality control standards

A series of calibration standards were made by spiking the mixed working standard solutions (10 μL) and the IS working solution (10 μL) in blank human plasma (200 μL) to yield concentrations of 0.538, 1.61, 5.38, 16.1, 32.3, 64.6, 129, 258 and 431 ng/mL for PPF and 0.511, 1.53, 5.11, 15.3, 30.7, 61.3, 123, 245 and 409 ng/mL for 5-OHP. The prepared low QC (LQC), medium QC (MQC) and high QC (HQC) samples were at the concentrations of 1.08, 10.8, 108 and 323 ng/mL for PPF, and 0.538, 1.61, 5.38, 16.1, 32.3, 64.6, 129, 258 and 431 ng/mL for 5-OHP.

Sample preparation

Frozen plasma samples were thawed at room temperature and thoroughly vortexed before analysis. An aliquot of 200 μL plasma sample and 10 μL IS solution were vortexed for 10 s. Then the mixture was precipitated with 800 μL methanol, vortex-mixed for 3 min and centrifuged at 15,600 rpm for 5 min. The supernatant (200 μL) was transferred to the autosampler vials and an aliquot of 5 μL was injected into the LC–MS-MS system for analysis.

Method validation

The method was validated for selectivity, linearity and sensitivity, LLOQ, accuracy and precision, recovery and matrix effect, carry-over effect, and stability according to the guidance published by the United States Food and Drug Administration (FDA) for bioanalytical method validation (22).
Selectivity
The selectivity was assessed by comparing chromatograms of blank human plasma from six different sources, blank human plasma spiked with two analytes at LLOQ and the IS, and a human plasma sample obtained from a volunteer after oral administration of a PPF-SR.

Linearity and sensitivity
There were both nine concentration levels on the calibration curves of PPF and 5-OHP. Each sample for calibration curves was freshly prepared. Linearity of the calibration curves, constructed by plotting the peak area ratios of analytes to the IS versus nominal concentrations, was investigated by weighted (1/x²) least-squares linear regression method.

The LLOQs are defined as the lowest analytical concentrations, which represent the sensitivity of the method. The analyte peaks in LLOQ samples should be identifiable, discrete and reproducible with acceptable precision (relative standard deviation (RSD) < 20.0%), accuracy (80.0–120.0%) and signal-to-noise ratio (S/N > 5).

Accuracy and precision
The accuracy and precision were evaluated of three validation batches over two days, each consisting of five replicated QC samples at three concentration levels (low, medium and high). The precision of the method including intra- and inter-batch precision was expressed as the RSD, which was required to be less than 15%. Accuracy was calculated as the relative error (RE), which was required to be within ±15%.

Recovery and matrix effect
Five replicates of QC samples at three QC concentration levels (low, medium and high) were prepared to assess recovery efficiency. Recovery was expressed as the ratio of peak areas obtained from extracted samples (spiked before extraction) to those of unextracted samples (spiked after extraction). The absolute matrix effect was evaluated by assaying six individual QC samples at three concentration levels, and was calculated as the ratio of peak areas of unextracted samples (spiked after extraction) to those of standard solutions prepared in mobile phase. Considering the matrix effects of the IS, normalized matrix factors (the ratio of the matrix factors of analytes to the matrix factor of the IS) were used to evaluate the relative matrix effect.

Carryover effect
Carryover effect was assessed by injecting blank samples after upper limit of the quantification (ULOQ) samples in five repeat runs to determine the interference at the retention time. It is required that the responses in the blank matrix at the retention times of the analytes and the IS should be <20% of LLOQs and 5% of the IS, respectively.

Stability
The stability of human plasma samples was assessed at low and high QC concentration levels in three replicates under different conditions: freeze–thaw stability after three freeze–thaw cycles between −20°C and room temperature; long-term stability at −20°C for 2 months; short-term stability at room temperature for 6 h; autosampler stability at 4°C for 27 h. In addition, the stability of stock solutions were evaluated at room temperature for 7 h and −20°C for 2 months. The analytes in human plasma samples were considered to be stable when the RE were all within 15% and the stock solutions were considered to be stable when the RE were all within 5%.

Application
The validated method was used to determine the plasma concentrations of PPF and 5-OHP in a pharmacokinetic study. In total, 10 healthy Chinese volunteers, aged 20–25 years, were admitted in the clinical site. The protocol was obedient to the ethical principles established in the Declaration of Helsinki and approved by the Ethics Committee of Nanjing Clinical Tech Laboratories Inc. (Nanjing, China), the permission number of clinical trials is CTLS201512 (expiration date: 15th October, 2016). All subjects underwent strict physical examinations, electrocardiograms and routine laboratory tests for health assessment. The volunteers were given a single oral administration of 425 mg PPF-SR with 250 mL water. Their median cubital vein was cannulated and 4 mL blood samples were collected before (0 h) and at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15, 24 and 36 h into sodium heparinized and labeled tubes after administration. Then the plasma samples were stored at −20°C after centrifugation at 4000 rpm for 10 min.

The pharmacokinetic parameters were calculated by Drug and Statistics Software (DAS®; professional edition version 3.2.7, Drug and Statistics, Shanghai, China) using non-compartmental methods. The pharmacokinetic parameters studied in this study were maximum plasma concentration (Cmax), the time to Cmax (Tmax), elimination half-life (t1/2), the area under the plasma concentration–time curve from 0 to 36 h (AUC0–36h) and the area under the plasma concentration–time curve from 0 to infinity (AUC0–∞).

Results
Method validation
Selectivity
Figure 1 shows the typical chromatograms of blank human plasma, blank human plasma sample spiked with two analytes at LLOQ and the IS, and a human plasma sample from a volunteer at 6.0 h after oral administration of a PPF-SR. No significant interference from the plasma was observed at the retention time.

Linearity and sensitivity
The calibration curves were linear over the range of 0.5–400 ng/mL for both analytes with correlation coefficient (r²) more than 0.997. The typical standard curves were f = 0.0194 × C + 0.0013 for PPF and f = 0.0136 × C + 0.0024 for 5-OHP, where f represents the peak area ratio of PPF or 5-OHP to the IS, and C represents the plasma nominal concentration of PPF or 5-OHP. The LLOQs were 0.5381 ng/mL for PPF and 0.5109 ng/mL for 5-OHP. The signal-to-noise ratios of the analytes at their LLOQs were much higher than 5, and the corresponding precision and accuracy of LLOQ samples were all within recommended limits (Table 1).

Accuracy and precision
The accuracy and precision of this method summarized in Table 1 shows that all the values of RSD and RE were all within recommended limits.

Recovery and matrix effect
It was proved that this method was reproducible by studying the recovery and matrix effect for both analytes. The recoveries were...
The recovery of the IS was 86.2%. Since there was no specific requirement for recovery according to the guidance published by the FDA for bioanalytical method validation (22) and the precision and accuracy values of QCs at three concentration levels were all within the acceptable criteria, indicating that the recovery of each analyte was consistent, precise, and reproducible.

The mean matrix effects of plasma were 94.3, 100.9 and 98.1% for PPF and 105.2, 98.5 and 102.0% for 5-OHP at three QC levels (1, 100 and 300 ng/mL), respectively. The mean matrix effects for the IS (1 μg/mL) were 98.5%. Considering the matrix effects of the IS, the mean values of IS-normalized matrix factors were 99.4, 100.6 and 99.6% for PPF and 102.6, 98.7 and 101.8% for 5-OHP at three QC levels (1, 100 and 300 ng/mL), indicating that there was no significant ion suppression or enhancement from plasma matrix for PPF and 5-OHP.

**Carryover effect**

During the experiment, very slight carryover effects can be observed for the two analytes and the IS in the blank plasma samples. However, these effects can be considered acceptable because the responses of the two analytes and the IS in the blank plasma samples were much less than the recommended limits of carryover effects.
Stability

Table II indicates that all analytes were stable in human plasma and processed samples under various conditions. The stock solutions of PPF, 5-OHP and IS at room temperature for 7 h and −20°C for 2 months were observed to remain stable.

Application

This proposed method was successfully applied to the pharmacokinetic study of PPF and 5-OHP in human plasma after oral administration of a PPF-SR (425 mg). The mean plasma concentration–time curves of PPF and 5-OHP are presented in Figure 2. The main pharmacokinetic parameters for ten healthy Chinese volunteers are shown in Table III. The results of our study indicate that the sustained-release capsule provides significantly slower release of PPF and 5-OHP compared with reported pharmacokinetic parameters of PPF and 5-OHP, which were all of instant-release formulations.

Discussion

In order to enhance the sensitivity and avoid the matrix effect, various mobile phases and the injection volumes were investigated. A series of aqueous mobile phases containing different additives (such as formic acid and ammonium acetate) were tested. A obvious conclusion can be drawn that lower background noise and less matrix effect would be possible when ammonium acetate was added into the aqueous part, which can provide better resolution of the analytes from endogenous interference materials. The use of formic acid in the aqueous part was to get better peak shape and mass response to the analytes. The methanol and acetonitrile were also on the investigated list for the organic mobile phase. Compared to acetonitrile, methanol demonstrates superiority over the mass responses to the analytes. Together these factors considered above, a mobile phase of methanol and 5 mM ammonium acetate solution containing 0.2% formic acid (pH 3.2) (68:32, v/v) became preferred. The flow rate of 0.4 mL/min produced appropriate retention times of PPF, 5-OHP and IS with high precision (Figure 1).

The mass spectrum conditions were then optimized and it was found that the best detection was produced when the parameters of drying gas flow, nebulizer pressure, drying gas temperature and capillary voltage, were set at 12 L/min, 50 psi, 350°C and 4000 V, respectively. The protonated molecular ions [M + H]+ at m/z 342.2, 358.2 and 384.2 were chosen as the parent ions for PPF, 5-OHP and IS, respectively. On the basis of that, the most abundant product ions at m/z 116.1, 116.2 and 253.1 were selected in the MRM transitions for PPF, 5-OHP and the IS, respectively (Figure 3).

### Table II. Stability of Propafenone and 5-Hydroxypropafenone in Human Plasma Under Various Storage Conditions (n = 3)

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Analytes</th>
<th>Concentration levels (mean ± SD, ng/mL)</th>
<th>RSD (%)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Added</td>
<td>Measured</td>
<td></td>
</tr>
<tr>
<td>Room temperature for 6 h</td>
<td>PPF</td>
<td>1.076</td>
<td>322.9</td>
<td>1.066 ± 0.022</td>
</tr>
<tr>
<td></td>
<td>322.9</td>
<td>325.7 ± 4.3</td>
<td>1.3</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>306.5</td>
<td>309.7 ± 6.1</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>Three freeze/thaw cycles</td>
<td>PPF</td>
<td>1.076</td>
<td>322.9</td>
<td>1.047 ± 0.049</td>
</tr>
<tr>
<td></td>
<td>322.9</td>
<td>329.2 ± 1.8</td>
<td>0.6</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>306.5</td>
<td>309.8 ± 1.7</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Autosampler for 27 h at 4°C</td>
<td>PPF</td>
<td>1.076</td>
<td>322.9</td>
<td>1.050 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>322.9</td>
<td>323.4 ± 5.9</td>
<td>1.8</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>306.5</td>
<td>306.4 ± 5.4</td>
<td>1.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Freezing for 60 days −20°C</td>
<td>PPF</td>
<td>1.076</td>
<td>322.9</td>
<td>1.001 ± 0.050</td>
</tr>
<tr>
<td></td>
<td>322.9</td>
<td>329.0 ± 0.6</td>
<td>0.2</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>306.5</td>
<td>310.4 ± 2.1</td>
<td>0.7</td>
<td>1.3</td>
</tr>
</tbody>
</table>

### Table III. Mean Pharmacokinetic Parameters of Propafenone and 5-Hydroxypropafenone in Human Plasma

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Parameters</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPF</td>
<td>Cmax (ng/ml)</td>
<td>210.9 ± 141.9</td>
</tr>
<tr>
<td></td>
<td>AUC0–36 (ng h/mL)</td>
<td>1610 ± 1309</td>
</tr>
<tr>
<td></td>
<td>AUC0–∞ (ng h/mL)</td>
<td>1620 ± 1314</td>
</tr>
<tr>
<td></td>
<td>t1/2 (h)</td>
<td>4.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>5-OHP</td>
<td>Cmax (ng/ml)</td>
<td>129.6 ± 65.4</td>
</tr>
<tr>
<td></td>
<td>AUC0–36 (ng h/mL)</td>
<td>1446 ± 754</td>
</tr>
<tr>
<td></td>
<td>AUC0–∞ (ng h/mL)</td>
<td>1522 ± 793</td>
</tr>
<tr>
<td></td>
<td>t1/2 (h)</td>
<td>7.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Tmax (h)</td>
<td>7 ± 2</td>
</tr>
</tbody>
</table>
preparation procedure and high sensitivity (0.5 ng/mL) for both two analytes. The pharmacokinetic parameters of the PPF-SR in healthy Chinese volunteers were reported for the first time. The devised method met all USFDA guidelines for validating a bioanalytical method (22).

**Conflit of interest statement.** The authors have declared no conflict of interest.

**References**


6. Barbery, J.T.; Clinical pharmacology and [beta]-blocking efficacy of propafenone; *Journal of Cardiovascular Pharmacology*, (1991); 17: S44.


17. Slawson, M.H., Johnson-Davis, K.L.; Quantitation of flecainide, mexiletine, propafenone, and amiodarone in serum or plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS); Methods in Molecular Biology, (2016); 1383: 11–19.


