A Validated HPLC–MS/MS Method for the Quantification of Supinoxin in Rat Plasma and Its Application to Pharmacokinetic Study

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A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the quantification of an anticancer drug, supinoxin (RX-5902), in rat plasma. Following precipitation pretreatment using 0.1% formic acid in acetonitrile, separation was performed using a reverse phase liquid chromatography column packed with C18 (3.5 μm, 2.1 × 50 mm) along with a mobile phase of 0.1% formic acid in distilled water and 0.1% formic acid in acetonitrile at a flow rate of 0.3 mL min⁻¹. Detection was achieved using MSMS by multiple reaction monitoring via an electrospray ionization source at mass/charge transitions of m/z 442.30 → 223.30 for supinoxin and m/z 430.08 → 223.20 for the internal standard DGG-200064. This method demonstrated a linear standard curve (r = 0.9980) over a supinoxin concentration range of 0.0005–1 μg mL⁻¹, as well as intra- and inter-assay precisions below 7.08% and 13.74%, respectively, and an accuracy of 1.15–4.50%. The matrix effect, recovery, and process efficiency were 93.63%, 99.70%, and 93.33%, respectively. Thus, a sensitive and reliable LC–MS/MS method was developed and validated for the quantification of supinoxin in rat plasma. This method was successfully applied to the evaluation of pharmacokinetic studies after single intravenous and oral administration of 1 mg kg⁻¹ supinoxin in rats.

Keywords: Supinoxin, RX-5902, LC–MS/MS, P-p68, pharmacokinetics

Introduction

Supinoxin (RX-5902) is an orally administered, first-in-class, small molecule inhibitor of phosphorylated-p68 RNA helicase (P-p68) [1]. Rexahn Pharmaceuticals is currently carrying out phase 1 clinical trials of supinoxin in cancer patients with solid tumors to evaluate the safety, tolerability, dose-limiting toxicities, and maximum tolerated dose [2, 3].

P68 (DDX5) is considered a prototypic member of the DEAD box family of RNA helicases. The DEAD box proteins are ribonucleic acid binding proteins with ATPase activity, and p68 has been reported to play an important role in the cell proliferation, differentiation, and metabolism of cancer cells [4–6]. P-p68 phosphorylated by tyrosine influences β-catenin to activate cyclin D1, c-JUN, and c-MYC, which promote the activity and differentiation of cancer [7–9]. Specifically, the phosphorylation of p68 at Y593 mediates the effects of platelet-derived growth factors in promoting epithelial–mesenchymal transition (EMT) and cell migration by facilitating β-catenin nuclear localization [8, 10]. The overexpression of P-p68 is frequently detected in solid tumors, such as malignant melanoma, colorectal cancer, ovarian cancer, and lung cancer [4, 11].

Supinoxin has been reported to inhibit P-p68 RNA helicase and prevent the transcription of cancer-related genes [12, 13]. In addition, it blocks the G2/M-specific cell cycle inhibition of the anti-apoptotic Bcl-2 protein by p21 induction [1]. Furthermore, supinoxin exhibited strong growth inhibition in various human cancer cell lines including breast, colon, pancreatic, ovarian, and stomach cancers, with IC₅₀ values ranging from 11 to 21 nM. Potent activity in drug-resistant cancer cells was also displayed. In mice bearing tumor xenografts, treatment with supinoxin completely inhibited the growth of various human tumors, such as melanomas, pancreatic, renal, and ovarian cancers, while enhancing tumor regression without affecting body weight when compared to control animals. Supinoxin also significantly inhibited the growth of drug-resistant cancer cells and has shown potential for use in combination therapy with known anticancer drugs, such as paclitaxel, doxorubicin, gemcitabine, fluorouracil, and cisplatin [14, 15].

Although supinoxin is in the final stages of phase 1 clinical trials, to date, no quantitative analytical method has been reported. In addition, very limited information is available regarding the pharmacokinetics of experimental animals (i.e., rats), which are generally used as reference data for drug discovery and for efficacy and toxicity studies. In this report, we outline a reliable analytical method for the quantification of supinoxin in rat plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS). This method was successfully used in the pharmacokinetic studies of supinoxin in rats and may be useful for the development of clinical data.

Experimental

Reagents and Materials. Supinoxin and the internal standard (IS) DGG-200064 were obtained from Dongguk University (Seoul, Korea; Figure 1). Acetonitrile and methanol (high-performance liquid chromatography [HPLC] grade) were purchased from Tedia Inc. (Fairfield, OH, USA). All other chemicals employed were either HPLC grade or the highest purity available. Rat plasma containing sodium heparin as an anticoagulant was prepared in-house.

HPLC Operating Conditions. HPLC was carried out using an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with an autosampler, a binary pump, a column...
Quantification of Supinoxin in Plasma with LC–MS/MS

Figure 1. Chemical structures of (a) supinoxin and (b) DGG-200064 (IS)

oven, and a system controller. Compounds were separated using a Zorbax XDB-C18 column (3.5 \( \mu \)m, 2.1 × 50 mm; Agilent Technologies) with an isocratic mobile phase comprising 0.1% formic acid in distilled water (DW) and 0.1% formic acid in acetonitrile (50:50 [v/v]) at a flow rate of 0.3 mL min \(^{-1}\). The column and autosampler tray were maintained at 25 °C and 4 °C, respectively. An injection volume of 5 \( \mu l \) and an analytical run time of 5 min were used.

**Mass Spectrometry Operating Conditions.** Quantification by MS/MS was carried out in positive mode for the analyte and IS using an Applied Biosystems/MDS SCIEX (Foster City, CA, USA) API 4000 Q-Trap mass spectrometer equipped with a turbo ion spray source. The ion spray voltage and temperature were 5500 V and 500 °C, respectively, while the curtain gas, nebulizer gas, and turbo gas pressures were 20, 40, and 40 psi, respectively. The entrance potential (EP), collision energies (CEs), and collision cell exit potential (CXP) for both supinoxin and the IS were 10, 23, and 14 V, respectively. The declustering potentials (DPs) were 16 and 86 V, respectively. A multiple reaction monitoring (MRM) mode was used for quantification at \( m/z \) 442.30 → 223.20 for supinoxin and \( m/z \) 430.08 → 223.20 for the IS. Data were analyzed by using Analyst software (version 1.4.1; Applied Biosystems/AB SCIEX).

**Preparation of the Calibration Standard, Quality Control, and Internal Standard Stock Solutions.** Primary stock solutions of 1 mg mL \(^{-1}\) supinoxin for use as the calibration standard and quality control (QC) samples were prepared in methanol. The stock solutions were stored at −20 °C and were diluted successively with acetonitrile to prepare working solutions containing supinoxin concentrations of 0.5, 2, 5, 10, 100, 300, and 1000 ng mL \(^{-1}\) for preparation of the calibration curve. In addition, working solutions of 1 ng mL \(^{-1}\) (low quality control, LQC), 30 ng mL \(^{-1}\) (medium quality control, MQC), and 900 ng mL \(^{-1}\) (high quality control, HQC) were prepared for QC. An IS working solution of 100 ng mL \(^{-1}\) was also prepared in acetonitrile.

**Sample Preparation.** To induce the precipitation of plasma proteins, the IS working solution (50 \( \mu l \)), the supinoxin working solution (50 \( \mu l \)), and 0.1% formic acid in acetonitrile (350 \( \mu l \)) were added to aliquots (50 \( \mu l \)) of the blank rat plasma to give the blank sample. In addition, the IS working solution (50 \( \mu l \)) and 0.1% formic acid in acetonitrile (400 \( \mu l \)) were added to aliquots (50 \( \mu l \)) of the rat plasma samples. The resulting mixture was mixed vigorously for 10 min and then centrifuged at 17,600 \( g \) for 10 min. Finally, a sample of the supernatant (200 \( \mu l \)) was placed in an autosampler vial, and an aliquot (5 \( \mu l \)) of this solution was injected directly into the LC–MS/MS system.

**Validation.** The full validation process, including the determination of selectivity, linearity, precision, accuracy, matrix effects, recovery, process efficiency, and stability, was performed in rat plasma according to Food and Drug Administration (FDA) guidance [16].

**Selectivity.** The selectivity of the method was evaluated by analyzing six different rat plasma matrices, zero samples (i.e., blank plasma containing the IS), and low limit of quantification (LLOQ) samples, to confirm the absence of potential endogenous interference in the chromatograms.

**Precision and accuracy.** To evaluate the precision and accuracy of the method, the intra-assay precision and accuracy were estimated by analyzing a batch consisting of three QC samples in six replicate (1, 30, and 900 ng mL \(^{-1}\) [17]). The intra-assay precision was determined by analyzing the three levels QC samples (LQC, MQC, and HQC) on three different days. The criteria for data acceptance were that the accuracy was within ±15% standard deviation (SD) from the nominal values and that the precision range was within ±15% relative standard deviation (RSD). The acceptance criteria for the method were that the LLOQ had a precision <20% of the RSD and an accuracy <20% of the relative error (RE).

**Matrix effects, recovery, and process efficiency.** Three articles were assessed by analyzing three sets (see below) of standards at three concentrations, namely, 1, 30, and 900 ng mL \(^{-1}\) [17]. Matrix effects for supinoxin and for the IS were assessed by comparing the mean analyte peak areas at three concentrations following extraction into blank plasma (set 2) with the mean peak areas for neat analyte solutions in the mobile phase (set 1). The recoveries of supinoxin and the IS by extraction were determined by comparing the mean analyte peak areas prior to extraction into the blank plasma (set 3) with set 2. The process efficiency was determined by comparing set 3 with set 1 [18, 19].

**Stability.** To evaluate the freeze–thaw cycle stability, short-term stability, and long-term stability, QC samples of low and high concentration (i.e., 1 and 900 ng mL \(^{-1}\), respectively) were assessed in quadruplicate. The freeze–thaw stability was determined using three freeze–thaw cycles. Short-term stability was determined by storing a sample at room temperature for 6 h prior to processing. Long-term stability was determined by storing four aliquots of the two QC sample concentrations (i.e., 1 and 900 ng mL \(^{-1}\), respectively) at −20 °C for 4 weeks.

**Application to Pharmacokinetic Studies.** The developed LC–MS/MS assay was applied in the pharmacokinetic study of supinoxin, whereby supinoxin was administered intravenously and orally to male Sprague–Dawley rats (age, 7 weeks; body weight, 190–210 g; Orient Bio, Seongnam, Korea). This study was conducted under the guidance of the Institutional Animal Care and Use Committee of Chungnam University (CNU-00398, Daejeon, Korea). For all experimental work, the animals were kept in plastic cages with free access to a standard rat diet (PMI Nutrition International, Richmond, IN, USA) and water at a temperature of 20–26 °C, with a 12 h light–dark cycle and a relative humidity of 40–60%. Prior to dosing, animals were...
fasted for 14 h but were allowed access to water. After dosing, food was provided after a further 4 h. Supinoxin was dissolved in a mixture containing 10% dimethyl sulfoxide, 40% polyethylene glycol 400, 10% Tween 80, and 40% saline, and administered through an IV bolus dose via the tail vein or an oral gavage dose (n = 4; dosing volume of 1 mL kg⁻¹ for all animals). Blood samples (300 μL) were obtained from the jugular vein at 0.083 (IV only), 0.25, 0.50, 1, 3, 7, 10, and 24 h after dosing. Blood samples were immediately centrifuged at 17,600g for 5 min, and the harvested plasma samples were stored at −20 °C until required for analysis.

The time course of drug plasma concentrations for each rat was analyzed by noncompartmental analysis using WinNonlin® 4.2 (Pharsight Corp., Cary, NC, USA). The peak plasma concentration (Cmax) and the time taken to reach the peak plasma concentration (Tmax) were obtained directly from the curves of plasma drug concentration vs. time. The elimination rate constant (Ke) was determined by linear regression of the log-linear portion of the terminal phase. The terminal elimination half-life (T1/2) was calculated by dividing ln2 by the previously determined Ke. The area under the plasma concentration vs. time curve from time zero to infinity (AUC₀–∞) was calculated using WinNonlin® 4.2 via the linear trapezoidal rule and the standard area extrapolation method. The systemic clearance (CL) and the distribution volume at a steady state (Vss) values for supinoxin were calculated according to the following equations:

\[
CL = \frac{Dose}{AUC_{0-\infty}} \tag{1}
\]

\[
V_{ss} = \frac{MRT \cdot CL}{(2)}
\]

\[
MRT = \frac{AUC_{0-\infty}}{CL} \tag{3}
\]

The values of Cmax and Tmax were obtained directly from the plasma concentration–time curves.

**Results and Discussion**

**Liquid Chromatography and Mass Spectrometry.** To obtain the optimal detection conditions for supinoxin and IS, MS/MS full scans were conducted in both positive and negative ion detection modes. In the positive ion mode, the response was appropriate for both supinoxin and IS. Using electrospray ionization (ESI), both supinoxin and IS were successfully ionized and produced predominantly protonated precursor molecules (MH⁺) at m/z 442.3 and 430.2, respectively. In the MS/MS compound optimization mode, each precursor ion was fragmented to yield the various product ions (see Figure 2). Based on the intensity of the product ions, those at m/z 223.2 for supinoxin and m/z 223.4 for IS were selected as the main product ions for the purpose of this study. Consequently, the precursor–product ion pairs of m/z 442.3 → 223.2 for supinoxin and m/z 430.2 → 223.4 for IS were selected for MRM analysis.

To optimize chromatographic separation, various chromatographic conditions were tested. As supinoxin and IS had similar hydrophobic scaffolds, a reverse phase C18 column was selected as the stationary phase. A mobile phase composed of 50% aqueous acetonitrile containing 0.1% formic acid at a flow rate of 0.3 mL min⁻¹ was selected for isocratic separation. Using these separation conditions, supinoxin and IS exhibited symmetrical peak shapes and sufficiently different retention times.

Figure 3 shows representative chromatograms of blank rat plasma, a plasma sample spiked with supinoxin (0.5 ng mL⁻¹), and a plasma sample obtained 3 h after the oral administration of supinoxin. The analysis of six different blank rat plasma samples showed no interference in the supinoxin and IS retention times (2.3 and 1.4 min, respectively). Based on these results, the selectivity of the analytical method was confirmed. Carryover was avoided by washing the needle with methanol for 5 s after each injection.

**Method Validation**

**Calibration curve.** The LLOQ was set at 0.5 ng mL⁻¹ for supinoxin using 50 μL of rat plasma. The signal-to-noise ratio for supinoxin was 10 times higher than the baseline at 0.5 ng mL⁻¹ (Figure 3b). In addition, standard calibration curves were obtained using eight calibration standards of rat plasma with supinoxin concentrations of 0.5–1000 ng mL⁻¹. Linear regression of the standard curve was fitted to \( y = ax + b \) (\( a = 0.000509, b = 0.0141, r = 0.9980 \)) using the weighting factor of \( 1/(\text{conc.}^2) \). Sensitivity was determined by analysis of the LLOQ samples (n = 8), and the precision and accuracy were determined based on the corresponding concentrations calculated at each level. Relative standard deviation (RSD) was within 3.3% to 7.8%, and the relative error (RE) ranged from 0.3% to 6.3%.

**Precision and accuracy.** Table 1 summarizes the precision and accuracy data for the intra- and inter-assays of the QC samples. Both assay values were within the accepted variable limit, with a precision range of 1.5–13.7% and an accuracy range of 0.17–11.60% at three QC concentrations.

**Matrix effect, recovery, and process efficiency.** The matrix effects oriented by the plasma components were 93.6% for supinoxin and 77.8% for the IS. Significant signal suppression was not observed for either the analyte or the IS. Recovery was examined for the 3 different QC mixtures, and 99.7% of both supinoxin and IS was recovered by protein precipitation. The overall process efficiencies of the protein precipitation method for both supinoxin and IS were 93.3% and 77.6%, respectively. These results indicate that precipitation is an appropriate pretreatment method for the extraction of supinoxin and IS from rat plasma.

**Stability.** The stability of supinoxin during sample handling between storage and analysis was evaluated using the LQC and HQC samples (Table 2). The stability over three freeze–thaw cycles and over both short-term and long-term storage was examined. The various sample preparation and storage conditions had little effect on the quantification of supinoxin in the LQC and HQC samples, and so the processed QC samples and calibration standards were allowed to stand at 4 °C for 24 h prior to injection, without affecting the quantification results.

**Dilution integrity.** The effects of sample dilution were examined for the QC samples that exceed the upper limit of quantification (ULOQ) of the calibration curve. Analysis of the 10× diluted samples was repeated in quadruplicate, demonstrating that the precision and accuracy were within the acceptable criteria.

**Application to Pharmacokinetic Studies.** The established analytical method was applied to a pharmacokinetic study following intravenous and oral administration of 1 mg kg⁻¹ supinoxin to four male rats. The mean plasma concentration vs. time profiles for supinoxin are shown in Figure 4. Following bolus intravenous injection, CL was 691 ± 110 mL min⁻¹ h kg⁻¹ and Vss was 2037 ± 316 mL kg⁻¹. AUC₀–∞ was 1.78 ± 0.30 μg h mL⁻¹, and T1/2 was 2.64 ± 0.39 h. For the oral gavage dose, the T1/2 and the AUC₀–∞ values were 2.11 ± 0.91 h and 1.01 ± 0.42 μg h mL⁻¹, respectively. Beyond Tmax, the plasma concentration–time profile corresponding to oral administration was comparable to that of intravenous administration. In addition, the oral bioavailability was 465
Figure 2. Product ion mass spectra of (a) supinoxin and (b) DGG-200064 (IS)
estimated by dividing \( \text{AUC}_{\text{oral}} \) by \( \text{AUC}_{\text{intravenous}} \), to give an absolute oral bioavailability of 56.86% (Table 3). Considering moderate bioavailability and low clearance, the intestinal absorption of supinoxin was considered marginal.

**Table 1.** Accuracy and precision of supinoxin determination

<table>
<thead>
<tr>
<th>Spiked concentration (ng mL(^{-1}))</th>
<th>Measured concentration (ng mL(^{-1}))</th>
<th>RE (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-batch ((n = 5))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.989</td>
<td>1.15</td>
<td>5.69</td>
</tr>
<tr>
<td>30</td>
<td>31.9</td>
<td>6.17</td>
<td>3.18</td>
</tr>
<tr>
<td>900</td>
<td>860</td>
<td>4.50</td>
<td>7.08</td>
</tr>
<tr>
<td>Inter-batch ((n = 3))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.17</td>
<td>13.74</td>
</tr>
<tr>
<td>30</td>
<td>31.5</td>
<td>4.39</td>
<td>7.05</td>
</tr>
<tr>
<td>900</td>
<td>884</td>
<td>11.60</td>
<td>1.53</td>
</tr>
</tbody>
</table>

Figure 3. HPLC–MS/MS chromatograms of samples from (a) double blank rat plasma, (b) rat plasma containing supinoxin (1 ng mL\(^{-1}\)) and DGG-200064 (1 \(\mu\)g mL\(^{-1}\)), and (c) a rat plasma sample obtained 3 h after oral administration.

**Conclusions**

In summary, we have developed and validated a rapid, simple, and sensitive LC–MS/MS method for the quantification of supinoxin in rat plasma. This method also involved sample
Table 2. Stability of supinoxin in rat plasma (n = 4)

<table>
<thead>
<tr>
<th>Stability conditions</th>
<th>Spiked concentration (ng mL⁻¹)</th>
<th>Measured concentration (ng mL⁻¹)</th>
<th>RE (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postoperative (24 h, 4 °C)</td>
<td>1</td>
<td>0.977</td>
<td>2.30</td>
<td>2.93</td>
</tr>
<tr>
<td>Short term (6 h, RT*)</td>
<td>1</td>
<td>846</td>
<td>5.95</td>
<td>2.35</td>
</tr>
<tr>
<td>Freeze-thaw (3 cycles)</td>
<td>1</td>
<td>1.02</td>
<td>2.09</td>
<td>2.24</td>
</tr>
<tr>
<td>Freezethaw (3 cycles)*</td>
<td>1</td>
<td>925</td>
<td>2.76</td>
<td>1.39</td>
</tr>
<tr>
<td>Freeze-thaw (3 cycles)</td>
<td>1</td>
<td>1.00</td>
<td>0.04</td>
<td>2.69</td>
</tr>
<tr>
<td>Long term (4 weeks, −20 °C)</td>
<td>1</td>
<td>900</td>
<td>0.03</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.958</td>
<td>0.41</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>900</td>
<td>895</td>
<td>1.53</td>
<td>1.77</td>
</tr>
</tbody>
</table>

*RT indicates room temperature.

Table 3. Pharmacokinetic parameters after a single IV or oral administration of supinoxin in rats

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Intraduvenous</th>
<th>Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_max (h)</td>
<td>0.083</td>
<td>0.650 ± 0.335</td>
</tr>
<tr>
<td>C_max (μg mL⁻¹)</td>
<td>0.860 ± 0.093</td>
<td>0.250 ± 0.093</td>
</tr>
<tr>
<td>AUC₂→∞ (μg h mL⁻¹)</td>
<td>2.64 ± 0.39</td>
<td>2.11 ± 0.91</td>
</tr>
<tr>
<td>AUC₀→∞ (μg h mL⁻¹)</td>
<td>1.76 ± 0.32</td>
<td>0.984 ± 0.407</td>
</tr>
<tr>
<td>CL (mL h⁻¹ kg⁻¹)</td>
<td>691 ± 110</td>
<td>1.01 ± 0.42</td>
</tr>
<tr>
<td>Vₘₚ (mL kg⁻¹)</td>
<td>2037 ± 316</td>
<td>56.86</td>
</tr>
</tbody>
</table>

T_max indicates time to reach the peak plasma concentration; C_max, peak plasma concentration; T₁/₂, elimination half-life; AUC, area under the plasma concentration–time curve; CL, systemic clearance; Vₘₚ, steady-state volume of distribution; BA, bioavailability.

preparation, and results demonstrated both adequate recovery and a lack of significant matrix effects. Finally, we successfully applied our method to a single-dose pharmacokinetic study in rats.

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References

Figure 4. Mean plasma concentration–time curves of supinoxin after intravenous (●) and oral (○) administration of supinoxin at 1 mg kg⁻¹ to rats. Each point represents the mean ± SD (n=4).