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Development and Validation of a Simple Method for the Detection of Fascaplysin in Plasma

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Abstract

Fascaplysin is a cytotoxic natural product isolated from a variety of Indo-Pacific marine organisms, primarily Fascaplysinopsis sponges and Didemnum tunicates. Positive xenograft studies involving this alkaloid structural class have indicated that fascaplysin may serve as an important lead compound for preclinical development. This study was undertaken as a prelude to a full pharmacokinetics and therapeutic assessment of fascaplysin. We describe here a simple plasma preparation and a rapid HPLC method for the detection of fascaplysin in mice. The method was validated by parameters including good linear correlation, a limit of quantification of 10.7 μg/mL, and a good precision with a coefficient of variation of 0.92% for 10 μg/mL. This method provides excellent sensitivity and visualization of fascaplysin as a single peak allowing for rapid analysis of plasma samples involving absorption, distribution, and metabolism studies. A preliminary pharmacokinetics study in C57Bl/6 mice using 20.6 mg/kg fascaplysin yielded a biphasic curve with T½α = 16.7 min, T½β = 11.7 h, and C0 of 17.1 μg/mL.

Introduction

Fascaplysin (1), a pigment first reported in 1988 from the marine sponge Fascaplysinopsis [1], is the parent compound of a uniquely marine-derived structural class (Figure 1). Most members of this family possess a framework consisting of a planar, fused-pentacyclic alkaloid often as a quaternary salt. To date 27 analogs of fascaplysin (1) have been reported [2-9] and several studies have described a broad range of bioactivity properties for this class including antiangiogenesis [10], antibacterial [1], antimalarial [11] and cytotoxicity against a range of bioactivity properties for this class including antiangiogenesis [10], antibacterial [1], antimalarial [11] and cytotoxicity against a range of bioactivity properties for this class including antiangiogenesis [10], antibacterial [1], antimalarial [11] and cytotoxicity against a range of bioactivity properties for this class including antiangiogenesis [10], antibacterial [1], antimalarial [11] and cytotoxicity against a.

Keywords: Fascaplysin; HPLC analysis; Plasma samples; Pharmacokinetics

Figure 1: Structures of the alkaloid salts, fascaplysin (1), and three analogs.

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in vivo bioactivity studies. We describe below a validated method to determine fascaplysin (1) concentration and stability in mouse plasma. The process consists of a simple extraction of plasma samples followed by isocratic HPLC analysis with UV detection of 1.

Materials and Methods

Instruments

A Waters Alliance HPLC system [including a Waters 2690 Separations Module, a Waters 2996 Photodiode Array Detector, a Waters Symmetry Shield RP 18 reversed-phase column (3.9×150 mm, 10 μm), and the Waters Empower software] (Milford, Massachusetts, USA), was used in this study for the determination of fascaplysin (1) in mouse plasma. A Beckman Coulter Allegra 21R Centrifuge (Fullerton, California, USA) was used to prepare the plasma and red blood cell samples for HPLC injection.

Reagents

Fascaplysin (1) (HPLC peak purity >99%) was isolated as previously described [3]. All solvents were HPLC grade and obtained from Burdick & Jackson (Muskegon, Michigan, USA).

Preparation of Plasma and Red Blood Cell (RBC) Samples

Mouse blood samples were collected by cardiac puncture using a 1mL syringe (with a 25-gauge needle) containing heparin as anticoagulant. Once the maximum amount of blood was collected, it was transferred to a 1.5 mL conical centrifuge tube and centrifuged at 14,000 rpm (equivalent to 19,000 g) at 4°C for 15 min to separate plasma and RBC. Plasma and RBC were separately transferred and stored in a freezer at -20°C. To prepare individual spiked standards, 20 μL of fascaplysin stock solutions in water were added to 230 μL of plasma samples, 500 μL of methanol was added to each, inverted and vortexed for 1 min, and centrifuged at 19,000 g at 4°C for 30 min. The supernatant was added to 2.0 ml of water and the mixture was passed through a Sep-Pak® Vac 1 cc (100 mg) C8 cartridge. Fascaplysin (1) was eluted with 1.0 mL of methanol and evaporated to dryness using an Eppendorf Vacufuge® at 45°C. 100 μL of mobile phase buffer was added to each sample. The same procedure was used to prepare RBC samples for HPLC analysis.

HPLC procedure

Fascaplysin (1) in the eluted fraction was determined using a Waters SymmetryShield RP18 reversed-phase column (3.9×150 mm) with a 5 μm particle size. The injection volume was 70 μL. The column oven was room temperature. The mobile phase was phosphoric acid/methanol/water (1/20/79 v/v/v) at a flow rate of 1.0 mL/min. Detection was done at 302 nm. The results of the analysis are expressed in nanograms of fascaplysin (1) per mL of plasma.

Calibration curve

A stock solution of 20 μg/mL of fascaplysin (1) was prepared in water. The calibration curve was obtained with mouse plasma spiked with known amounts of the stock solution. The analyte was extracted according to the protocol described above. The calibration curve was obtained by plotting the peak area of fascaplysin (1) against its concentration in 7 spiked plasma standards (20, 10, 1, 0.5, 0.25, 0.1, and 0.05 μg/ml) and performing a linear regression analysis.

Pharmacokinetics study

Fascaplysin (1) was administered i.v. to C57Bl/6 female mice at 20.6 mg/kg in a volume of 0.25 mL saline, containing 5% DMSO and 5% Cremophor/Propylene glycol (60/40, v/v); two animals per time point were sacrificed at 5 min., 0.5, 1, 2, 4, and 24 hr after drug administration. Blood samples were collected and analyzed as described above. Animal treatment was in full accordance with the Institutional Animal Care and Use Committee (IACUC) Guidelines for the care and management of laboratory animals.

Results and Discussion

The data gathered allows detection of fascaplysin (1) over a wide range of concentrations. The method developed here was validated according to the U.S. Pharmacopoeia [23] and the analytical performance parameters considered were selectivity, linearity, accuracy, precision, sensitivity, ruggedness, and peak symmetry [24].

Somewhat similar to a past study [25], seven parameters are used to validate the method and the analytical details are as follows:

Selectivity

The absorption spectrum for fascaplysin (1) (Figure 2)
the identity of the chromatographic peak for fascaplysin (1) both by its
confirmed that fascaplysin (1) was well-resolved and free from interference peaks.
typical chromatogram at 302 nm (Figure 3) for spiked plasma indicated
302.6, and 408.8 nm. In all further studies, 302 nm was chosen. A
were obtained by running six replicates of each standard for a full
indicating that the method is precise (Table 2) [26].
Calibration curves of fascaplysin (1) were prepared to determine the
linearity of the method over the range of 50-20,000 ng/mL. Integrated
peak areas were plotted against analyte concentration, and linear
regression was performed by the least-squares method. The calibration
curve generated using plasma as matrix showed a square of correlation
coefficient of 0.998 (Figure 4).

The accuracy of the analytical method was determined by application of the method to analytes of known concentrations. Three spiked plasma samples (0.25, 10 and 20 μg/mL) were prepared in triplicate and analyzed. The assay results were 0.253, 9.82, and 20.01 ng/mL, indicating a recovery of 100.4%, 98.2%, and 101.2%, respectively for these samples. Therefore, the method is accurate (Table 1).

The precision of the analytical method was determined by analyzing a plasma sample spiked with 10 μg/mL of fascaplysin (1) injected ten times. Peak areas were considered for the determination. The precision, expressed as the percent coefficient of variation (CV), was 0.92% indicating that the method is precise (Table 2) [26].

The limit of detection (LOD) and the limit of quantification (LOQ) were obtained by running six replicates of each standard for a full standard curve. A linear regression was performed on each standard curve and the standard deviation of the y-intercepts was computed. The LOD was computed by multiplying the standard deviation by 3.3, adding that number to the average y-intercept value and dividing by the average slope (Table 5). The LOD for this method is 0.019 μg/ml and the LOQ is 0.107 μg/ml.

Ruggedness
Ruggedness was shown by assaying 3 replicates of spiked plasma samples using 3 fascaplysin (1) concentrations on two consecutive days employing the corresponding linearity curve generated on the day the sample was assayed. As shown in (Table 3), intra- and inter-day assay results of a particular sample are essentially the same, indicating an acceptable ruggedness of the method for intra- and inter-day assay studies. In addition, three spiked samples (100, 250, and 500 ng/mL) were analyzed using slightly modified flow rates. As shown in (Table 4), when the flow rate was reduced or increased to 0.9 or 1.1 mL/min, respectively, as compared to the normal 1mL/min. The assay results showed CV values of 2.1%, 2.0%, and 0.6%, further indicating that the present method is rugged with respect to minor changes of the flow rate.

Peak symmetry
Tailing factor is defined as the distance from the front slope of the
peak to the back slope (AB) divided by twice the distance from the
average slope. Similarly, the LOQ was computed by multiplying the standard deviation by 10, adding that number to the average y-intercept value and dividing by the average slope (Table 5). The LOD for this method is 0.019 μg/ml and the LOQ is 0.107 μg/ml.

Table 1: Average peak areas of three fascaplysin (1) standard concentrations and percent recoveries (n=3).

<table>
<thead>
<tr>
<th>Fascaplysin (µg/ml)</th>
<th>Average Peak Area</th>
<th>Average Computed Concentration</th>
<th>Average % Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>1023</td>
<td>0.253</td>
<td>100.4</td>
</tr>
<tr>
<td>10</td>
<td>50725</td>
<td>9.82</td>
<td>98.2</td>
</tr>
<tr>
<td>20</td>
<td>104033</td>
<td>20.01</td>
<td>101.2</td>
</tr>
</tbody>
</table>

Table 2: Replicate precision in the peak area determinations for 10 μg/ml fascaplysin (1) concentration.

<table>
<thead>
<tr>
<th>Day 1 Replicate</th>
<th>Peak Area</th>
<th>Average</th>
<th>S.D.</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>100171</td>
<td>108079</td>
<td>104033</td>
<td>3954</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>49472</td>
<td>50855</td>
<td>51848</td>
<td>50725</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>1052</td>
<td>997</td>
<td>1020</td>
<td>1023</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 2 Replicate</th>
<th>Peak Area</th>
<th>Average</th>
<th>S.D.</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate 1</td>
<td>103268</td>
<td>99336</td>
<td>102708</td>
<td>101770</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>50027</td>
<td>49735</td>
<td>50486</td>
<td>50083</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>1011</td>
<td>997</td>
<td>1022</td>
<td>1010</td>
</tr>
</tbody>
</table>

Table 3: Assay of a fascaplysin (1) standard sample over a period of 2 days.

<table>
<thead>
<tr>
<th>Effect Flow Rate Variation on Assay (ng/mL)</th>
<th>Flow Rate (mL/min)</th>
<th>Change Level</th>
<th>100 ng/mL</th>
<th>250 ng/mL</th>
<th>500 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>100171</td>
<td>-0.1</td>
<td>101</td>
<td>243</td>
<td>501</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>98</td>
<td>249</td>
<td>506</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>+0.1</td>
<td>97</td>
<td>253</td>
<td>499</td>
<td></td>
</tr>
<tr>
<td>Avg ± SD</td>
<td>99 ± 2.08</td>
<td>258 ± 5.03</td>
<td>501 ± 3.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.1</td>
<td>2.0</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Assay results of three samples with known concentrations under different flow rates.
50 ng/mL at 24 hr. The T½α (17 min) was calculated between 5 min and 30 min, decreasing to 1809 ng/mL at 30 min, 351 ng/mL at 2 hr and then shown in (Figure 6), where the plasma level at 5 min was 14,533 ng/mL, indicating that the peak symmetry is good.

A preliminary PK assessment was carried out in C57Bl/6 mice injected i.v. with 20.6 mg/kg (0.4 mg/mouse) of 1. The results are shown in (Figure 6), where the plasma level at 5 min was 14,533 ng/mL, decreasing to 1809 ng/mL at 30 min, 351 ng/mL at 2 hr and then 50 ng/mL at 24 hr. The T½α (17 min) was calculated between 5 min and 30 min; T½β (11.7 hr) was calculated between 60 min and 1440 min; Cmax was 17.1 μg/mL. Fascaplysin (1) levels in red blood cells (RBC) were also analyzed; 3.4 μg/mL at 5 min decreasing to 127 ng/mL at 2 hr; after 2 hr, the RBC concentration of fascaplysin (1) was not detectable.

In summary, we have shown that each of the seven parameters discussed above provide a good basis for further biological evaluation of fascaplysin (1). The method described herein is suitable for analyzing plasma samples of fascaplysin (1). We have successfully developed a simple method to determine fascaplysin (1) in plasma, and present for the first time the absorption values of this compound in mouse blood from 5 min to 24 hr. Until now, no data have been published concerning absorption, bioavailability, and tissue distribution of fascaplysin (1). Future studies will evaluate both the pharmacokinetics and therapeutic efficacy of fascaplysin (1) in tumor-bearing mice.

**Acknowledgment**

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**References**


