Quantitative Determination of AVI-7100, a Phosphorodiamidate Morpholino Oligomer (PMOplus®), in Human Plasma Using LC-MS/MS

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Introduction

Phosphorodiamidate Morpholino Oligomers (PMOs) are exchanged synthetic oligonucleotides comprised of 2'-modified morpholino rings connected by phosphorodiamidate linkages (Figure 1). This modification of the 5'-monophosphate ends and phosphodiester linkages in natural RNA or DNA renders the PMO stable in the presence of nucleases and prevents any innate immune response. The nucleotide sequence can be easily designed to target specific DNA or RNA sequences allowing directed silencing or inhibition of transcription or translation.

AVI-7100 is a positively charged phosphorodiamidate morpholino oligomer (PMOplus®). Positive charges are introduced by addition of pendant piperazinyl phosphorodiamidate linkages replacing certain phosphorothioate neutral linkages and the positive charges serve to increase binding to the negatively charged target gene sequence. AVI-7100 is designed to bind to viral RNAs and inhibits the synthesis of the M1 and M2 matrix proteins. These proteins are involved in viral replication, assembly and budding processes. Inhibition of the M1 and M2 matrix proteins is intended to disrupt viral replication and slow the spread of the disease to other cells in the body. Further, the ability to target the immune system regulaory pathways may also enable broad-spectrum activity against multiple viral infections.

A hybridization-based LC-fluorescence assay was previously reported to quantify a 23mer neutral PMO (AVI-436) in rat plasma with a linear range of 4 to 6,500 ng/mL, but with a run time of 30 minutes per sample. Here we report a more sensitive, specific and high-throughput LC-MS/MS assay for the accurate quantitation of AVI-7100 in human plasma samples with a linear range of 5-1,000 ng/mL and a cycle time of 13 minutes.

Methodology

Sample Preparation and Extraction

1. Add 200 µL of plasma sample into the corresponding wells of a 96-well deep well plate.

2. Add 200 µL of 1% FA in water to a final flow rate.

3. Add 200 µL of 1% ammonia acetate buffer.

4. Wash the samples with 200 µL of 100 mM ammonium acetate buffer.

5. Wash the samples with 200 µL of a mix of FA, H 2O and ACN.

6. Elute the samples with 2 x 25.0 µL of a mix of FA, H 2O and ACN.

Chromatographic Conditions

Column: Venusil XT-ODS 120Å 5 micron (5 µm, 2.1x150 mm)

Flow rate: 0.5 µm/min (back flush mode with mobile phase C at 1.0 mL/min)

Gradient type: Linear/Isocratic

SRR transitions: AVI-7100 (M+H)+ 885.6 and 859.7 which represent the molecules carrying m/z 885.8 and 859.7 respectively. The analyte and the IS still slightly overlap, however, the back-calculated concentrations from their nominal values were within ± 7.0% and the relative standard deviation was 4.8%. For MRM monitoring of AVI-7100 and AVI-4225, respectively. The analyte and the IS still slightly overlap; however, the back-calculated concentrations from their nominal values were within ± 7.0% and the relative standard deviation was 4.8%.

Table 1. Back-Calculated Concentrations of Calibration Standards for AVI-7100

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>AVI-7100 (M+H)+ 885.8 Calibration</th>
<th>AVI-7100 (M+H)+ 859.7 Calibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.00</td>
<td>4.57</td>
<td>5.10</td>
</tr>
<tr>
<td>25.00</td>
<td>6.87</td>
<td>7.10</td>
</tr>
<tr>
<td>100.00</td>
<td>12.12</td>
<td>12.10</td>
</tr>
<tr>
<td>500.00</td>
<td>60.05</td>
<td>60.06</td>
</tr>
</tbody>
</table>

Table 2. Quality Control Samples for IntraAssay Accuracy and Precision for AVI-7100

<table>
<thead>
<tr>
<th>QC Level</th>
<th>AVI-7100 (M+H)+ 885.8 Calibration</th>
<th>AVI-7100 (M+H)+ 859.7 Calibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>1.50</td>
<td>1.55</td>
</tr>
<tr>
<td>Medium</td>
<td>5.00</td>
<td>5.10</td>
</tr>
<tr>
<td>High</td>
<td>25.00</td>
<td>25.10</td>
</tr>
</tbody>
</table>

Figure 1. Core structure of PMOs.

Figure 2. 3D Scan of AVI-7100.

Figure 3. Product ion scan spectrum of precursor ion of AVI-7100 (M+H)+ 885.6.

Results and Discussion

Method Development

MS Tuning

The molecular weights of AVI-7100 and AVI-4225 are 745.1 and 859.7 respectively. The most intense precursor ions for AVI-7100 and AVI-4225 are m/z 885.6 and 859.7 which represent the molecules carrying m/z 885.8 and 859.7 respectively. For AVI-7100, we observed product ion scan spectrum with the retention times of 111.5-129.5; which are from cytosine, thymine, guanine, and adenine respectively. To avoid matrix and analyte interferences, m/z transitions 885.6 — 859.7 and 859.7 — 825.2 were selected for MRM monitoring of AVI-7100 and AVI-4225 respectively. The analysis and the IS still slightly overlap, but contributes each other with the selected MRM transitions, therefore, chromatography conditions were developed to readily separate the analytes and IS peaks.

Optimization of LC Conditions and Extraction

For LC development, challenges that were encountered and overcome included poor chromatography, low selectivity and carryover. In order to achieve acceptable chromatography, various HPLC columns were screened; only the Varian Metasil AQ-C18 column provided acceptable resolution and peak shape. Various mobile phases were screened; it was determined that a buffer with methanol and acetonitrile provided the sharpest peaks. Carryover from injector and column were observed during method development; or in order to minimize the ion suppressor, a strong needle wash solvent containing TEA, GS, and SMH (1 µM) was chosen. Carrier compounds were bombarded by back flushing the columns with a strong solvent containing FA, GS, and SMH at a high flow rate.

To optimize the extraction conditions and recovery, a screening test was performed following the Aneskal® sample extraction protocol using a proprietary 96-well plate. A µL SPE extraction yielded the highest recovery and best precision for both analytes.

Assay Linearity

AVI-7100 and AVI-4225 are very water soluble; however, significant compound loss was observed when pure aqueous neat solutions (Figure 4). Therefore, all solutions and samples were prepared and stored in polypropylene containers. Even in polypropylene containers, significant sample loss was observed when pure aqueous neat solutions were stored at room temperature.

Table 3. Matrix Factor Results for AVI-7100 and AVI-4225 (IS)

<table>
<thead>
<tr>
<th>Matrix Factor</th>
<th>AVI-7100</th>
<th>AVI-4225</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-Medium QC</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Medium QC</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>High QC</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Even in polypropylene containers, significant sample loss was observed when pure aqueous neat solutions were stored at room temperature.

Figure 5. Representative chromatogram of low QC sample.

Figure 6. Representative chromatogram of low standard (5.00 ng/mL).

Figure 7. Representative standard calibration curve (5-1000 ng/mL).