GLP Validation in Guinea Pig Plasma of an HPLC/MS/MS Method for the Quantitative Analysis of Pyridostigmine, a Pretreatment Compound for Chemical Warfare Agents

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Objectives
- Develop and validate GLP HPLC/MS/MS method to determine low concentrations of pyridostigmine in guinea pig plasma
- Simplify extraction to precipitation and direct injection method
- Optimize MS signal by using HPLC conditions with high organic concentrations of mobile phase

Introduction
Pyridostigmine Bromide (PB) is a pretreatment drug used to protect against chemical warfare nerve agents.

Since the guinea pig PK experiments required the use of less than 30 µL of plasma with quantitation limits near 100 pg/mL, a highly sensitive analytical method was needed such as HPLC/MS/MS. Previous methods developed at Alturas (1) had limits of quantitation of only 500 pg/mL. Thus improvements in the method are needed.

Internal standard is deuterated (D6) pyridostigmine.

Pyrido-MS Signal Vs. % ACN

HPLC Challenges
- PB is highly polar thus difficult with reversed-phase HPLC
- PB is a quaternary amine thus interacts with silanol groups from silica based columns to cause tailing
- Ion-pair reagents or high concentrations of aqueous phase needed for reversed-phase HPLC decreases ESI-MS signal

Methods

Extraction
- Precipitation of plasma proteins from 25 µL of guinea pig plasma in 96 well plates
- Extract (50 µL ACN) and transfer supernant
- Directly inject 30 µL of supernatant onto HPLC column

HPLC
- Strong-Cation Exchange
- 70% ACN/30% 100 mM ammonium formate
- Flow rate = 0.6 mL/minute
- PolyLC SCX poly (2sulfoethyl aspartamide) (2 x 35 mm)
- Thirty µL injections

Mass Spectrometry
- Sciex API3000 operating in MRM mode
- Turboionspray (400 °C)
- Positive ion mode
- MRM transitions –
  - 8180 ➔ 124 (PB)
  - 8186 ➔ 130 (D6 Internal Standard)

Pyridostigmine Standard (0.1ng/mL) from Guinea Pig Plasma

Previous Method HPLC/MS/MS Chromatogram for the Analysis of a Pyridostigmine Standard (0.50ng/mL) from Guinea Pig Plasma

New Method HPLC/MS/MS Chromatogram for the Analysis of a Pyridostigmine Standard (0.1ng/mL) from Guinea Pig Plasma

Table 1. QC Results for the HPLC/MS/MS GLP Validation of Pyridostigmine from Guinea Pig Plasma.

<table>
<thead>
<tr>
<th>QC Level (ng/mL)</th>
<th>% Accuracy</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLOQ-0.100</td>
<td>101</td>
<td>6.28</td>
</tr>
<tr>
<td>0.300</td>
<td>92.4</td>
<td>9.10</td>
</tr>
<tr>
<td>10.0</td>
<td>101</td>
<td>9.07</td>
</tr>
<tr>
<td>40.0</td>
<td>113</td>
<td>6.58</td>
</tr>
<tr>
<td>ULOQ-50.0</td>
<td>93.5</td>
<td>6.82</td>
</tr>
</tbody>
</table>

Conclusions
- Validated HPLC/MS/MS method to quantitate pyridostigmine from guinea pig plasma
- HPLC improvements show >5X signal increase compared to previous methods
- Method is simple, rapid, precise and accurate
- Pyrido short-term stability studies
  - Stable through 3 freeze-thaw cycles
  - Stable on bench top in plasma

References