

A Novel Method for the HPLC/MS/MS Quantitative Analysis in Guinea Pig and Mouse Plasma of Pyridostigmine, a Pretreatment Compound for Chemical Warfare Agents

Shane Needham¹, Binying Ye¹, J. Richard Smith² and William Korte³

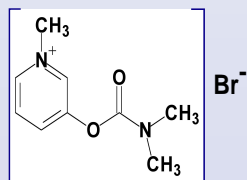
1. Alturas Analytics, Inc., Moscow, ID 2. U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD
3. Department of Chemistry, California State University, Chico, CA

Overview

• **Purpose** - Develop an HPLC/MS/MS method to determine low concentrations of pyridostigmine in guinea pig and mouse plasma

• **Methods** - 96 well-plate extraction and HPLC/ESI/MS/MS (API3000)

• **Results** - Range from 0.5 to 100 ng/mL with accuracies and precision better than 15% using only 25 μ L of sample



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 if needed for reversed-phase HPLC may decrease ESI-MS signal

Methods

Extraction

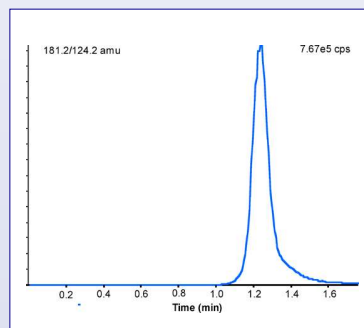
- Precipitation of plasma proteins from 25 μ L of guinea pig or mouse plasma in 96 well plates
- Extract, transfer supernant, evaporate and reconstitue in 100 μ L of mobile phase

HPLC

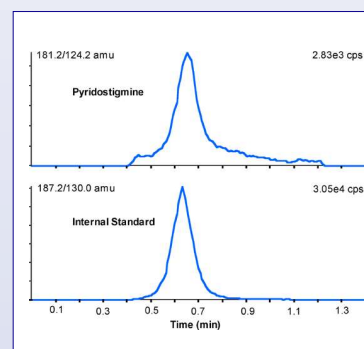
- 2% ACN in Water with 0.1% TFA and 1 mM ammonium acetate
- Flow rate = 0.6 mL/minute
- PRP 1 (Analytical Sales and Service) poly(styrene-divinylbenzene) 2.1 x 50 mm column
- Forty μ L injections

Mass Spectrometry

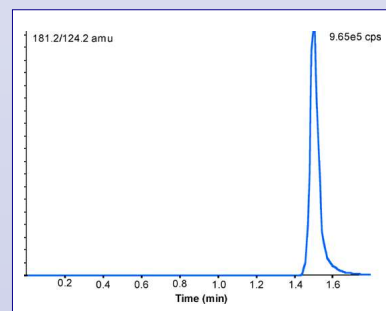
- Sciex API3000 operating in MRM mode
- Turboionspray (400 $^{\circ}$ C)
- Positive ion mode
- MRM transitions -
 - 181 \rightarrow 124 (PB)
 - 187 \rightarrow 130 (D6 Internal Standard)



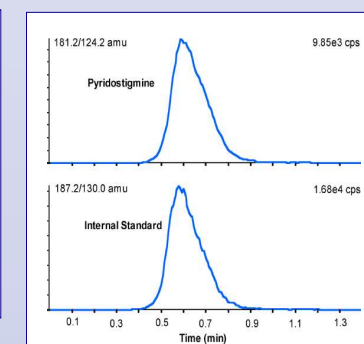
HPLC/MS/MS Chromatogram from the Analysis of a Pyridostigmine Standard Using a PRP Column without TFA.



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



HPLC/MS/MS Chromatogram from the Analysis of a Pyridostigmine Standard Using a PRP Column with the Addition of 0.1% TFA.



HPLC/MS/MS Chromatogram from the Analysis of a Pyridostigmine Sample from Mouse Plasma (Calc. Conc. 3.6 ng/mL).

Table 1. Standard Curve Results for the HPLC/MS/MS Analysis of Pyridostigmine from Guinea Pig Plasma.

Standard Curve Level (ng/mL)	Calculated Concentration (ng/mL)	% Accuracy
0.50	0.486	97.2
0.50	0.502	100.4
0.70	0.716	102.3
1.0	0.940	94.0
1.0	1.06	105.5
5.0	5.08	101.6
5.0	5.47	109.3
50	43.0	86.1
50	51.1	102.3
100	100	100.2
100	101	101.1

Introduction

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

Since the guinea pig and mouse PK experiments required the use of less than 30 μ L of plasma with quantitation limits near 500 pg/mL, a highly sensitive analytical method was needed such as HPLC/MS/MS. Previous methods employed HPLC/MS, however, the method required 50 μ L of plasma with a limit of quantitation of 700 pg/mL. In addition, the use of single ion monitoring subjected the assay to interferences.

Conclusions

- Developed HPLC/MS/MS method to quantitate pyridostigmine from guinea pig and mouse plasma
- Method is rapid, precise and accurate
- Future work
 - Full GLP validation
 - Improve LLOQ by at least 2x with changes in chromatography
 - Further simplify sample preparation