LC-MS/MS Analysis of Edrophonium, Neostigmine, and Pyridostigmine in Plasma Using HILIC Chromatography and Weak Cation-Exchange SPE

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Application Note 20787

Key Words
SOLA WCX, weak ion exchange, mixed mode, SPE, Syncronis HILIC, edrophonium, neostigmine, pyridostigmine, benzyldimethylphenylammonium chloride

Abstract
A liquid chromatography-tandem mass spectrometry method for the analysis of edrophonium, neostigmine, and pyridostigmine in plasma has been developed. Analysis was carried out using Thermo Scientific™ SOLA™ WCX solid phase extraction (SPE) products, a Thermo Scientific™ Syncronis™ hydrophilic interaction liquid chromatography (HILIC) UHPLC column for separation, and a Thermo Scientific™ TSQ Vantage™ mass spectrometer for MS/MS detection.

The selectivity of SOLA WCX cartridges or plates allows fast and simple extraction of quaternary ammonium ions from plasma with excellent reproducibility and precision, low matrix effects, and good recovery. Fast analysis was achieved with the Thermo Scientific Syncronis HILIC 1.7 µm column with a cycle time of less than 3 minutes.

Introduction
Edrophonium (Figure 1), neostigmine (Figure 2), and pyridostigmine (Figure 3) are acetylcholinesterase inhibitors. These compounds function as reversible competitive or noncompetitive inhibitors of cholinesterase and can be used to treat myasthenia gravis, an autoimmune disorder whose main symptom is muscle fatigue.

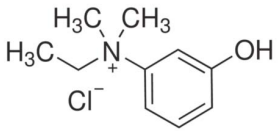

Figure 1: Edrophonium

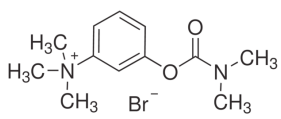

Figure 2: Neostigmine

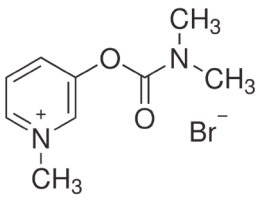

Figure 3: Pyridostigmine

SOLA WCX mixed-mode polymeric weak cation exchange products introduce additional selectivity into the SOLA SPE range. The SOLA SPE product range introduces next generation, innovative technological advancements, giving unparalleled performance characteristics compared to conventional SPE, phospholipid, and protein precipitation products.

These include:

- Higher levels of reproducibility
- Reduced sample and solvent requirements
- Higher levels of extract cleanliness
- Increased sensitivity

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SOLA SPE plates or cartridges provide significant advantages when analyzing compounds in complex matrices, particularly in high-throughput bioanalytical and clinical research laboratories where reduced failure rates, higher analysis speed, and lower sample/solvent requirements are critical. SOLA products' superior performance gives higher confidence in analytical results and lowers cost without compromising ease-of-use or requiring complex method development.

Edrophonium, neostigmine, and pyridostigmine are all quaternary ammonium ions and are characterized by remaining ionized across the whole pH range. This makes extraction using strong ion exchange mixed-mode SPE challenging as pH cannot be used to disrupt the interaction between the stationary phase and the analyte. Extractions can be achieved by using salts as competitive counter ions; however, the high concentration of salts in the elution solvent can be undesirable, particularly for LC-MS/MS analysis. Mixed-mode weak ion exchange SPE utilizes a hydrophobic backbone for reversed-phase interaction and a weak acid or base functionality on the sorbent for ion exchange. This weak ion exchange moiety allows ion exchange extraction to take place because the ionization of the stationary phase can be controlled by pH adjustment. As a result, it is possible to develop simple and effective SPE methods for the analysis of permanently charged analytes with additional matrix cleanliness, especially when used in mixed-mode format.

One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential to obtain this goal. The Syncronis column range provides exceptional reproducibility with highly pure, high surface area silica, dense bonding, and double endcapping, all controlled and characterized through the use of rigorous testing. The zwitterionic nature of Syncronis HILIC makes it ideal for the retention of small, highly polar quaternary ammonium ions, such as edrophonium, neostigmine, and pyridostigmine, which are not retained using a reversed-phase C18 column.

Experimental Details

Consumables	Part Number
Fisher Scientific™ LC/MS grade water	W/011217
Fisher Scientific LC/MS grade methanol	M/4062/17
Fisher Scientific LC/MS grade acetonitrile	A/0626/17
Fisher Scientific analytical grade formic acid	F/1900/PB08
Fisher Scientific ammonium formate	A/5080/53
Thermo Scientific™ Acros Organics™ sodium phosphate dibasic anhydrous	10440481

Sample Handling Equipment	Part Number
Thermo Scientific™ HyperSep™ glass manifold 24 port	60104-233
Stopcocks for 24 port manifold	60104-244
Vacuum pump	60104-241
Thermo Scientific™ UltraVap™ high speed sample concentrator	CLS-229070
Thermo Scientific™ eVol™ Sample Dispensing system	66002-024
Thermo Scientific™ Finnpipette™ F2 pipettor kit	PMP-020-220F
Vials and closures: Convenience kit, Wide Open Short Screw SureStop Vial, Clear with ID Patch, GOLD Grade and Red PTFE/White Silicone Septum	2-SVWGKST-CPK

Preparation of reference solutions, standards and QC samples

Standard spiking solutions of edrophonium, neostigmine, and pyridostigmine were prepared in acetonitrile / ammonium formate, 100 mM, pH 3.3 (80:20 v/v). A working internal standard (benzyldimethylphenylammonium chloride) was prepared in acetonitrile / ammonium formate, 100 mM, pH 3.3 (80:20 v/v). Spiking solutions for each level of calibrant were prepared in acetonitrile / ammonium formate, 100 mM, pH 3.3 (80:20 v/v).

180 µL of blank plasma was added to a vial. For standards and quality control (QC) samples, 10 µL of standard spiking solution and 10 µL internal standard were added. For blanks, 20 µL of acetonitrile / ammonium formate, 100 mM, pH 3.3 (80:20 v/v) was added. To all samples 800 µL of sodium phosphate, dibasic, 20 mM, pH 8 was added and samples were vortex mixed for 30 s.

Sample Preparation		Part Number
Compounds:	Edrophonium, neostigmine, pyridostigmine, and benzyldimethylphenylammonium chloride (IS)	
Matrix:	Plasma	
Cartridge type:	SOLA WCX 10 mg 1 mL cartridge	60109-004
Conditioning stage:	Apply 500 µL methanol + 5% formic acid, then 500 µL methanol, and then 500 µL sodium phosphate, dibasic, 20 mM, pH 8 sequentially to the SPE cartridges	
Application stage:	Apply all the sample to the SPE cartridge at a flow rate of 0.5 mL/min	
Washing stage:	Apply 500 µL sodium phosphate, dibasic, 20 mM, pH 8 to the SPE cartridge, then apply 500 µL methanol to the SPE cartridge	
Elution stage:	Apply 2 x 250 µL methanol + 5% formic acid to the SPE cartridge	

Dry under nitrogen and reconstitute in 500 µL acetonitrile / ammonium formate, 100 mM, pH 3.3 (80:20 v/v). Mix well. As sensitivity was easily achieved, a 2.5 fold dilution was performed to ensure solubility of the compounds in the reconstitution solvent.

Separation Conditions		Part Number
Instrumentation:	Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC HPLC system	
Column:	Syncronis HILIC 1.7 µm, 100 mm × 2.1 mm	97502-102130
Column protection:	UHPLC Filter	22180
	UHPLC Direct Connect Filter Holder	27006
Mobile phase:	Acetonitrile / ammonium formate, 100 mM, pH 3.3 (90:10 v/v)	
Flow rate:	500 µL/min	
Column temperature:	25 °C	
Pressure:	210 bar	
Injection volume:	2 µL	

MS Conditions	
Instrumentation:	TSQ Vantage MS
Ionization conditions:	HESI
Polarity:	Positive
Spray voltage:	3000 V
Vaporizer temperature:	275 °C
Sheath gas pressure:	30 Arb
Aux gas pressure:	20 Arb
Capillary temperature:	300 °C
Collision pressure:	1.5 mTorr
Scan time:	0.02 s
Q1:	0.7 FWHM
Q3:	0.7 FWHM

Compound transition details are provided in Table 1.

Compound	Parent (m/z)	S-Lens (V)	Product (m/z)	Collision Energy (V)
Edrophonium	165.8	67	136.1	25
Neostigmine	222.9	95	208.1	20
Pyridostigmine	180.9	72	72.1	20
Benzyldimethylphenylammonium chloride	212.2	60	120.1	31

Table 1: Compound transition details

Data Processing	
Software:	Thermo Scientific™ LCQUAN™ version 2.6

Results

Chromatography

The analysis was performed on a Syncronis HILIC 1.7 µm UHPLC column. As shown in Figure 4, the three compounds and internal standard were separated in less than 3 minutes.

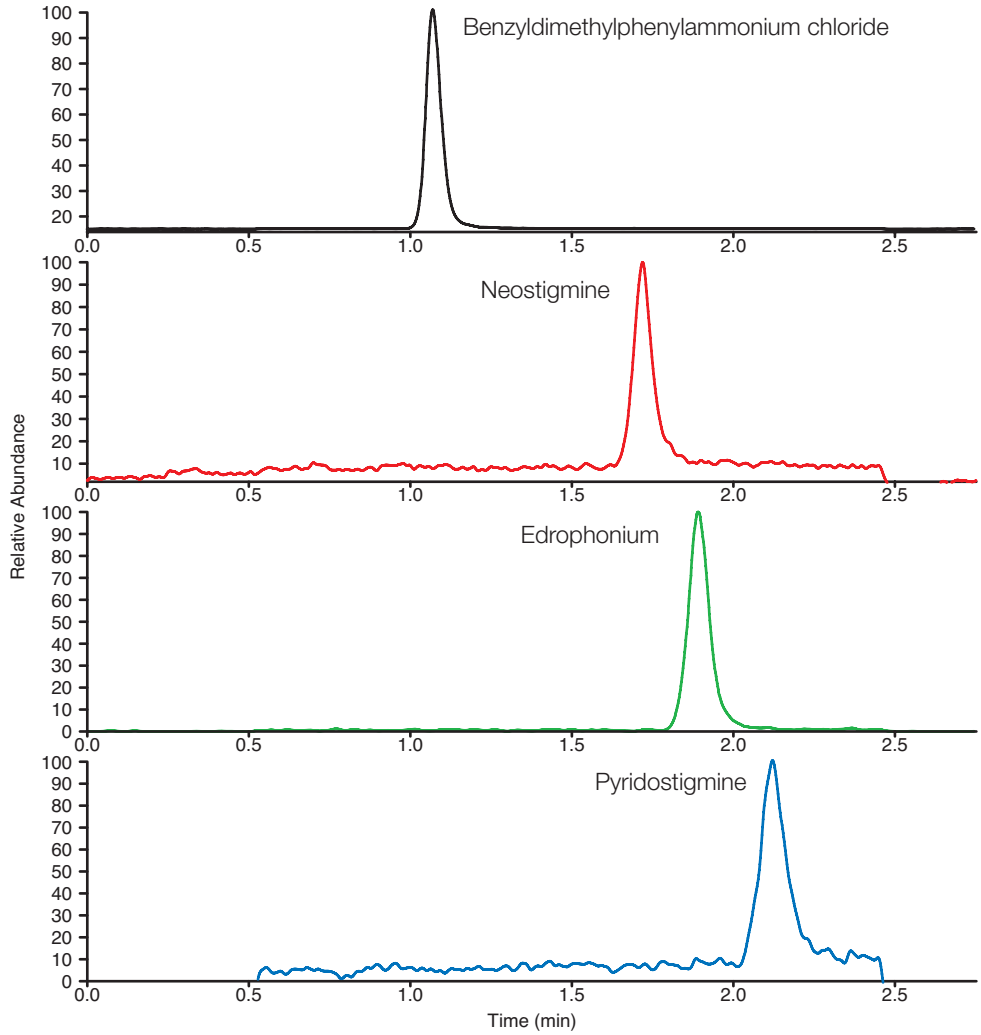

Figure 4: Representative chromatograms of benzyldimethylphenylammonium chloride, neostigmine, edrophonium, and pyridostigmine, extracted from plasma at 0.3 ng/mL

Linearity

Standards of edrophonium, neostigmine, and pyridostigmine extracted from spiked plasma gave a linear calibration curve over the dynamic range of 0.1 to 100 ng/mL with an r^2 of 0.9985 for edrophonium, 0.9972 for neostigmine, and 0.9968 for pyridostigmine (Figures 5, 6, and 7, and Table 2).

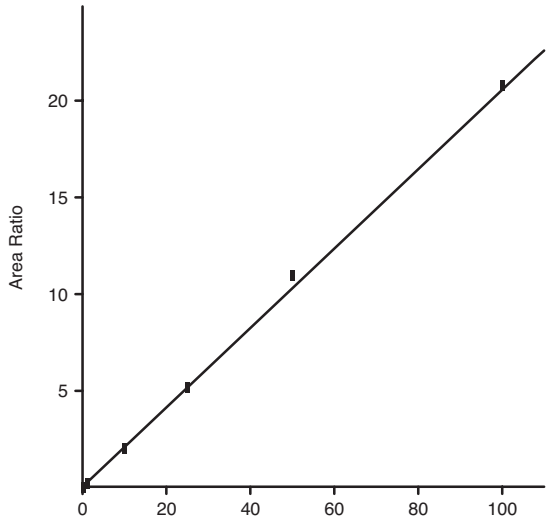

Figure 5: Edrophonium linearity over the dynamic range of 0.1 to 100 ng/mL

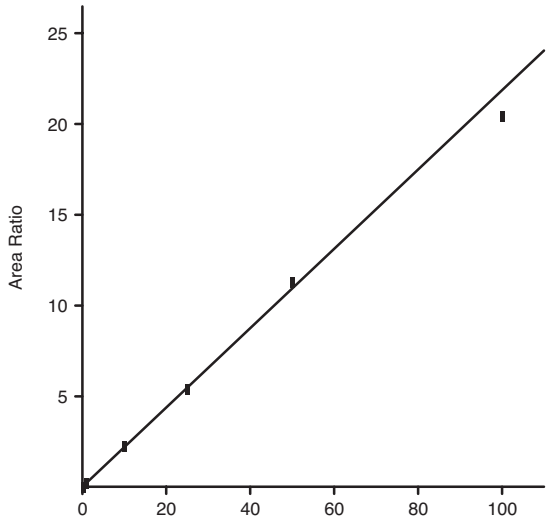

Figure 6: Neostigmine linearity over the dynamic range of 0.1 to 100 ng/mL

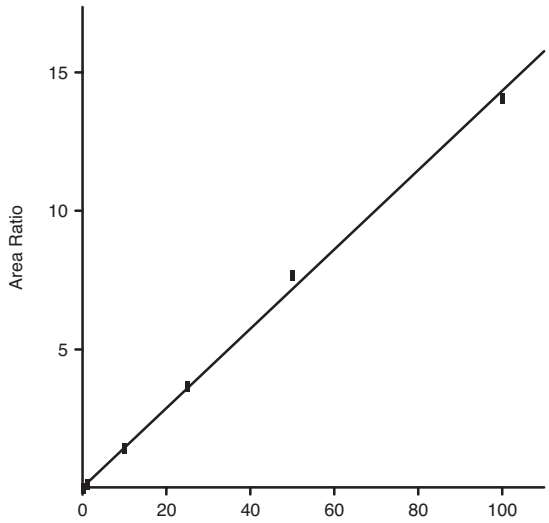

Figure 7: Pyridostigmine linearity over the dynamic range of 0.1 to 100 ng/mL

Accuracy and Precision

Standard	Specified Concentration (ng/mL)	Calculated Concentration Edrophonium (ng/mL)	% Difference Edrophonium	Calculated Concentration Neostigmine (ng/mL)	% Difference Neostigmine	Calculated Concentration Pyridostigmine (ng/mL)	% Difference Pyridostigmine
S1	0.1	0.100	-0.17	0.101	0.89	0.100	0.42
S2	1	1.01	1.42	0.910	-9.00	0.957	-4.28
S3	10	10.4	3.52	9.92	-0.83	9.96	-0.43
S4	25	24.7	-1.20	25.2	0.75	25.4	1.41
S5	50	51.5	3.07	53.5	6.98	52.9	5.81
S6	100	93.4	-6.64	101	1.22	97.1	-2.94

Table 2: Accuracy of back-calculated values for edrophonium, neostigmine, and pyridostigmine extracted standards over the linear range of 0.1 to 100 ng/mL

Replicate extractions (n=20) at the 0.3 ng/mL level were performed to assess the reproducibility of the assay. The %CV was calculated at less than 7% for the three compounds with no internal standard correction and less than 6% for all three compounds when using internal standard correction (Table 3). This illustrates excellent reproducibility of the assay even without an internal standard.

Standard	Concentration (ng/mL)	Average Calculated Concentration (n=20)	Precision with IS Correction %CV	Precision without IS Correction %CV
Edrophonium	0.3	0.31	5.6	7.0
Neostigmine	0.3	0.27	3.9	5.8
Pyridostigmine	0.3	0.29	4.3	5.7

Table 3: Average precision data for 20 replicate low QCs (0.3 ng/mL) for edrophonium, neostigmine, and pyridostigmine

Recovery

Overspikes (post-extracted fortified blanks) were run in triplicate at 0.3 ng/mL and used to calculate the percentage recovery level for edrophonium, neostigmine, and pyridostigmine (Table 4). The recovery was greater than 70% for all three compounds.

Standard	Response	% Recovery [i]
Edrophonium average QC response	8831	90.9
Edrophonium average overspike response	9712	
Neostigmine average QC response	8679	74.9
Neostigmine average overspike response	11592	
Pyridostigmine average QC response	5423	80.8
Pyridostigmine average overspike response	6373	

Table 4: Recovery data for edrophonium, neostigmine and pyridostigmine

Matrix Suppression

Overspikes and solution standards were run in triplicate at 0.3 ng/mL and used to calculate the % matrix suppression for edrophonium, neostigmine, and pyridostigmine (Table 5). There was very little matrix suppression: 0.1% for edrophonium, 1.5% for neostigmine, and -7.6% for pyridostigmine. The matrix effects were consistently low throughout the batch for all compounds.

Standard	Response	Absolute % Matrix Suppression [ii]
Edrophonium average overspike response	9712	0.1
Edrophonium average solution response	9856	
Neostigmine average overspike response	11937	1.5
Neostigmine average solution response	11608	
Pyridostigmine average overspike response	6373	-7.6
Pyridostigmine average solution response	5921	

Table 5: Matrix suppression data for edrophonium, neostigmine, and pyridostigmine

[i] $$\text{Absolute Recovery} = \frac{\text{average response of sample}}{\text{average response of overspike}} \times 100$$

[ii] $$\text{Absolute \% Matrix Suppression} = 100 - \frac{\text{average response of overspike}}{\text{average response of solution standard}} \times 100$$

Conclusion

- SOLA WCX chemistry allows for the fast and easy extraction and quantification of quaternary ammonium ions edrophonium, neostigmine, and pyridostigmine from plasma.
- Extraction recoveries were 90.9%, 74.9%, and 80.8% for edrophonium, neostigmine, and pyridostigmine, respectively.
- The SOLA WCX cartridge gave excellent precision for the extraction with %CV (n=20) less than 7% for all compounds, even without internal standard correction.
- The SOLA WCX cartridge achieved low absolute matrix suppression effects at less than 8% for all compounds.
- An LLOQ of 0.1 ng/mL was achieved.
- The zwitterionic bonding on Syncronis HILIC columns enabled the retention and separation of these quaternary ammonium analytes.
- Using a 1.7 µm particle size, a fast method with a run time of less than three minutes was developed.

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