APPLICATION NOTE 22054

Quantitation of acetylcholinesterase inhibitors

Utilizing the high loading capacity of SOLA WCX 30 mg in plasma for clinical research

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Goals

- Develop a sensitive ultra-high-performance liquid chromatography-tandem mass spectrometer (UHPLC-MS/MS) method for several acetylcholinesterase inhibitors in human plasma, utilizing the high load-ability of the Thermo Scientific™ SOLA™ 30 mg WCX SPE phase
- Ensure assay has low matrix effects and high recovery for all analytes
- Demonstrate the high separation performance and excellent peak shapes provided by the Thermo Scientific™ Accucore™ Polar Premium LC column for the analysis of samples from plasma

Introduction

This application note describes the use of the Thermo Scientific™ SOLA™ 30 mg cartridges to achieve an exceptionally sensitive assay in the range of 0.050–50 ng/mL for neostigmine, pyridostigmine, and edrophonium (Figure 1) due to the high loading capacity of the SOLA sorbent material. SOLA is a revolutionary form of solid phase extraction (SPE) that incorporates a

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fritless polymeric sorbent and is produced using advanced packing techniques. This means that it removes the issues commonly associated with conventional SPE (Figure 2 and Figure 3). The removal of these issues results in higher levels of reproducibility in processing viscous biological samples by reducing blocking and sample failures.

Acetylcholinesterase inhibitors are a group of compounds that block the breakdown of acetylcholine into choline and acetate by inhibiting the acetylcholinesterase enzyme. This is important because it increases both the level and duration of acetylcholine's effects within the central nervous system, autonomic ganglia, and the neuromuscular junctions.

Some of these compounds occur naturally, such as onchidal, which is produced as a defensive secretion by the mollusc *Onchidella binneyi* and acts irreversibly with the same mechanism as many of the deadly synthetic nerve agents.

More importantly, some of these compounds may be used medicinally to treat various neuromuscular diseases such as myasthenia gravis (MG). MG is a chronic autoimmune neuromuscular disease that leads to varying degrees of skeletal muscle weakness. It is caused by antibodies blocking or destroying the nicotinic acetylcholine receptors at the junction between nerve and muscle cells. This action prevents the nerve impulses from reaching the muscles to generate a contraction.

Symptomatic benefits can be provided by acetylcholinesterase inhibitors, such as those analyzed in this assay, but they may not fully remove a person's weakness caused by MG. The assay analytes neostigmine, pyridostigmine, and edrophonium are shown in Figure 1.

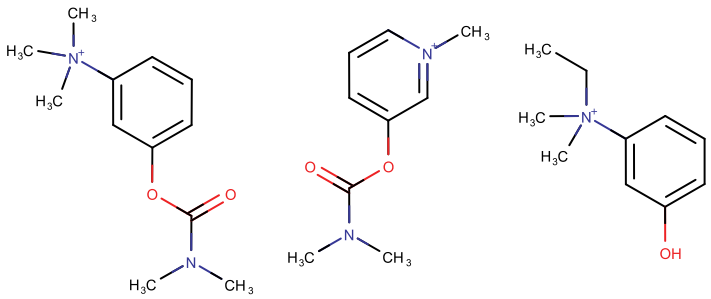

Figure 1. Molecular structures of neostigmine, pyridostigmine, and edrophonium (from left to right)

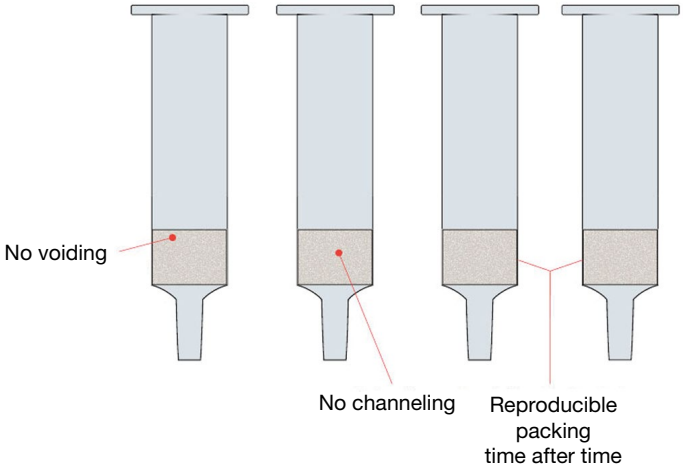

Figure 2. SOLA cartridge, fritless technology

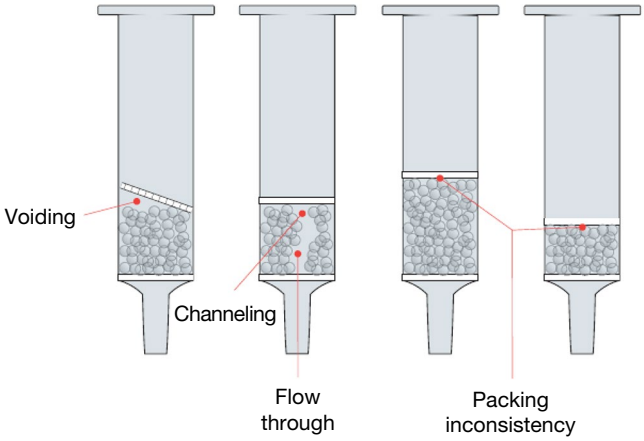

Figure 3. A generic silica SPE cartridge, packed with frits

Experimental

Consumables

Instruments

- Thermo Scientific™ Vanquish™ Horizon UHPLC System (IQLAAAGABHFAPUMZZZ) with the following modules:
  - System base Vanquish Horizon (P/N VH-S01-A)
  - Binary pump H (P/N VH-P10-A)
  - Split sampler HT (P/N VH-A10-A)
  - Column compartment H (P/N VH-C10-A)
  - Active pre-heater (P/N 6732.0110)
- Thermo Scientific™ TSQ Altis™ Triple Quadrupole Mass Spectrometer (TSQ02-10002)
- Thermo Scientific™ HyperSep™ Glass 24 Port Vacuum Manifold (P/N 60104-233)

Table 1. Consumables list

Product name	Part number
SOLA WCX 30 mg/3 mL cartridges, 50 pack	60409-004
Accucore Polar Premium column 100 × 2.1 mm, 2.6 µm	28026-102130
Thermo Scientific™ UHPLC-MS grade water	W8-1
Fisher Chemical™ Optima™ UHPLC-MS grade methanol	A456-212
Fisher Chemical™ Optima™ UHPLC-MS grade ammonium formate	A115-50
Fisher Chemical™ Optima™ UHPLC-MS grade formic acid	10596814
Thermo Scientific™ Chromacol™ GOLD-Grade 2 mL Short Thread SureStop™ Inert Vial	2-SVWGK
Fisherbrand™ SureOne™ Micropoint Pipette Tips, Universal Fit, Non-Filtered	10492725
Fisherbrand™ SureOne™ Micropoint Pipette Tips, Universal Fit, Non-Filtered	10003414

Sample preparation

1. Add 500 µL of human plasma to the bottom of the 96-well plate well.
2. Dilute by adding 1000 µL of water to the well (adjusted to pH 7 with ammonia and formic acid) and vortex.
3. Conditioning: Add 1000 µL of acetonitrile/formic acid (100/5, v/v) to the SPE cartridge and apply a pulse of vacuum to pull through and discard the collection.
4. Followed by 1000 µL of methanol; again applying a pulse of vacuum to pull though the solvent and discard the collection.
5. For the final conditioning step add 1000 µL of water (adjusted to pH 7 with ammonia and formic acid) to the SOLA WCX 30 mg/3 mL cartridge and apply vacuum to pull through so that the sorbent is still wet while again discarding the collected solvent.
6. Load: Apply the whole sample onto the SPE plate and apply vacuum to pull through slowly, at approximately 1 drip per second. When complete, discard the collected fluid.
7. Wash 1: Add 1000 µL of water (adjusted to pH 7 with ammonia and formic acid) to the SPE wells and apply vacuum to pull through slowly. Discard the collection.
8. Wash 2: Add 1000 µL of methanol to the well and apply vacuum slowly until sorbent is dry, discard the solvent collected.
9. Elute: Elute with 2 × 500 µL of ACN/formic acid (100/5, v/v) under vacuum at 1 drip per second until sorbent is dry; Collect both elutions in the same tube and carry onto the next step.
10. Evaporate to dryness under nitrogen at 50 °C.
11. Reconstitute in 250 µL of 100 mM ammonium formate/HCOOH (100/0.5, v/v).
12. inject onto the LC system.

Chromatographic conditions

Table 2 shows the chromatographic conditions used and Table 3 shows the gradient ramp conditions for the LC system.

Table 2. Chromatographic conditions

Parameter	Value
Run time	3.25 min
Column temperature	30 °C
Injection volume	1.5 µL
Mobile phase A	100 mM ammonium formate/HCOOH (100/0.5, v/v)
Mobile phase B	MeOH/HCOOH/DFA (100/0.5/0.1, v/v/v)

Table 3. Gradient ramp conditions

Time	Flow (mL/min)	%B
0.00	0.75	0
0.75	0.75	0
2.00	0.75	60
2.00	0.75	100
2.50	0.75	100
2.50	0.75	0
3.25	0.75	0

Mass spectrometry conditions

Table 4 shows the ion source conditions used for this assay while Table 5 shows the SRM parameters selected for mass spectrometer. Table 6 provides the SRM table for the quantification and confirming ions used for the analytes.

Table 4. Ion source conditions

Parameter	Value
Ion source type	H-ESI
Polarity	Positive
Voltage	+3500 V
Sheath gas	60 Arb
Aux gas	14 Arb
Sweep gas	2 Arb
Ion transfer tube temperature	325 °C
Vaporizer temperature	350 °C

Table 5. SRM properties

Parameter	Value
Dwell time	15 ms
Q1 resolution (FWHM)	0.2
Q3 resolution (FWHM)	0.2
CID gas	1.5 mTorr
Source fragmentation	20 V

Results and discussion

Chromatography

The Accucore Polar Premium column demonstrates outstanding performance for the separation of these three analytes and the internal standard used. Excellent peak shape and peak height was observed as shown in Figure 4, which allowed quantitation down to 50 pg/mL in a short run time of 3.25 min.

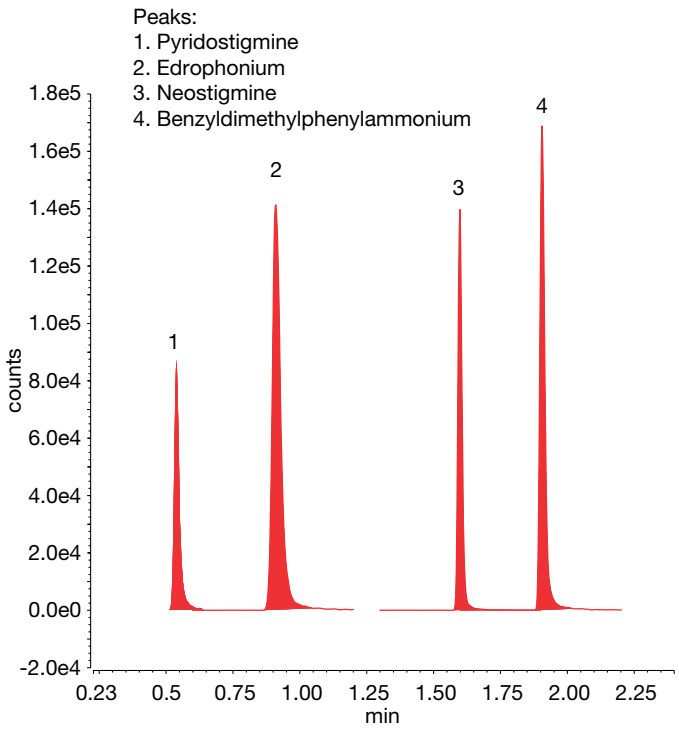

Figure 4. Chromatogram at MQC level

Table 6. SRM table

Compound	Precursor (*m/z*)	Product (*m/z*)	Collision energy (V)	RF lens (V)
Edrophonium^	166.161	136.07	27.23	49
Edrophonium*	166.161	137.05	18.64	49
Pyridostigmine^	181.161	72.03	18.63	47
Pyridostigmine*	181.161	124.07	14.61	47
Benzyldimethylphenylammonium(IStd)*	212.175	120.05	14.14	32
Benzyldimethylphenylammonium(IStd)^	212.175	121.1	15.95	32
Neostigmine^	223.175	72.07	28.87	63
Neostigmine*	223.175	208.1	19.16	63

^ = quantitation ion, * = confirming ion

Accucore columns contain solid core particles that are engineered to a diameter of 2.6 µm, within a narrow particle size distribution. This allows for several benefits, including the ability to achieve high speed and high resolution separations while operating at significantly lower backpressures compared to a fully porous equivalent using a smaller particle size.

The Accucore Polar Premium column is a rugged amide embedded C18 phase that offers complementary selectivity to the conventional C18 phase but is also compatible with 100% aqueous phases.

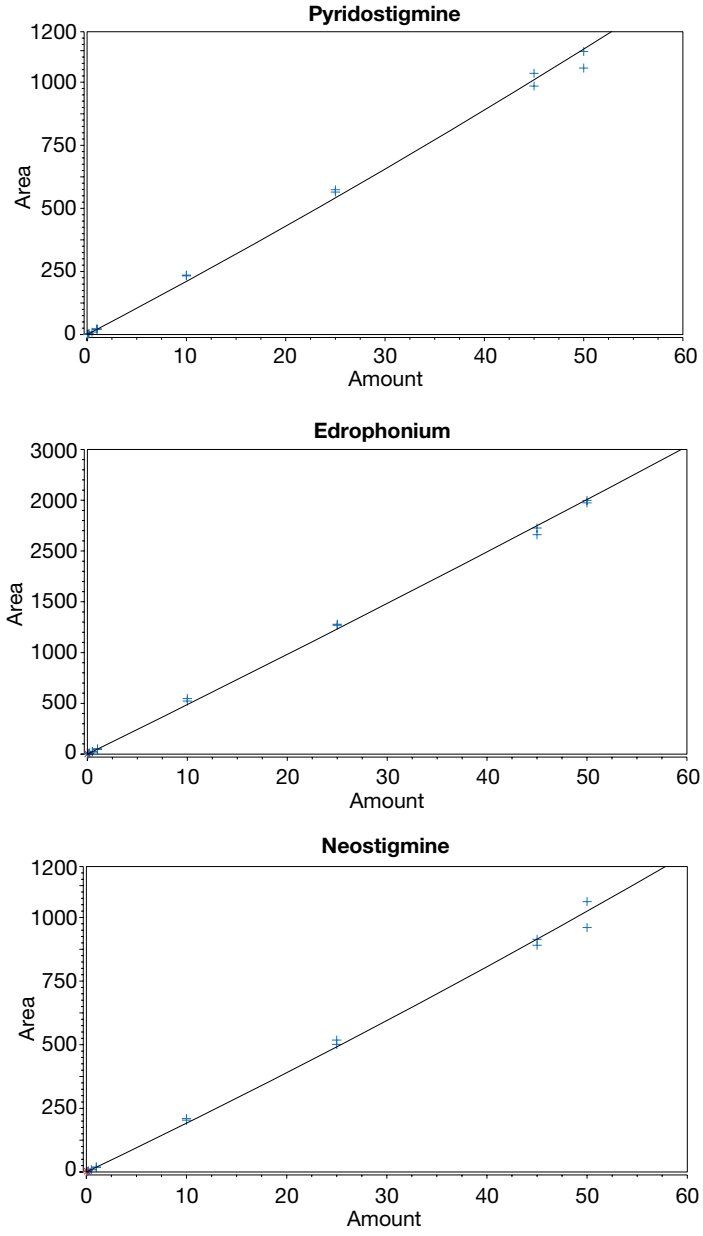

Figure 5. Calibration lines for the three analytes: pyridostigmine, edrophonium, and neostigmine

Calibration lines

All three analytes showed excellent R^2 values with 0.995, 0.994, and 0.996 for pyridostigmine, edrophonium, and neostigmine, respectively. The calibration type for all analytes was linear and used 1/response^2 weighting. The calibration lines are shown in Figure 5 for all three analytes.

Accuracy and precision

Data in Tables 6, 7, and 8 show the mean values of six individual samples for each QC level per analyte. The accuracy (bias %) in the table was well within the acceptance criteria for the assay which was set at ±15% of the nominal value at all QC levels. Additionally, excellent precision (CV%) was achieved across all QC levels, being <8.3% for all.

Table 6. Pyridostigmine QC results

	LLOQ	LQC	MQC	HQC
Target	0.0500	0.150	3.00	40.0
Mean	0.0530	0.161	3.33	44.2
Bias %	6.00	7.30	10.9	10.6
CV %	4.60	4.80	3.00	2.40

Table 7. Edrophonium QC results

	LLOQ	LQC	MQC	HQC
Target	0.0500	0.150	3.00	40.0
Mean	0.0510	0.161	3.34	43.9
Bias %	2.00	7.30	11.2	9.70
CV %	3.80	5.70	1.60	1.30

Table 8. Neostigmine QC results

	LLOQ	LQC	MQC	HQC
Target	0.0500	0.150	3.00	40.0
Mean	0.0460	0.134	2.63	42.9
Bias %	-8.00	-10.7	-12.2	7.30
CV %	8.30	1.20	2.70	1.10

Matrix effects

This assay has demonstrated very low matrix effects as a result of the fantastic selectivity of the SPE phase and method for the analytes of interest and the high-resolution separation provided by the Accucore Polar Premium phase. The matrix effects were assessed by bringing blank matrix through the sample clean-up and post spiking after using a solution made up to the expected final concentration. The signal response was then compared with a non-extracted sample of the same concentration to give the matrix effects as shown in Table 9.

Table 9. Matrix effects data for all analytes

Sample name	Mean analyte peak area	Analyte matrix factor	Mean IS peak area	IS matrix factor	IS normalized factor
Pyridostigmine MQC extract	123831	1.06	218692	0.966	1.09
Pyridostigmine MQC non-extract	117283		226370		
Edrophonium MQC extract	315473	1.02	218692	0.966	1.05
Edrophonium MQC non-extract	310385		226370		
Neostigmine MQC extract	147069	1.02	218692	0.966	1.06
Neostigmine MQC non-extract	143870		226370		

Recovery

The assay demonstrated excellent recovery for all analytes as shown in Table 10. Pyridostigmine, edrophonium, neostigmine, and benzyldimethylphenylammonium had great recoveries thanks to the selectivity of the SOLA WCX 30 mg/3 mL SPE cartridge, which allows the analytical method to be adjusted to target specific analytes as was performed in this assay.

Table 10. Recovery data for the assay

Analyte name	Recovery (%)
Pyridostigmine	104.8
Edrophonium	91.8
Neostigmine	91.2
Benzyldimethylphenylammonium	95.8

Discussion

For this assay the analytes all had a quaternary amine that was permanently charged over the full pH range. This led to the choice of using the SOLA WCX phase for the sample preparation as the weak cation exchangers charge can be switched on (charged) and off (no-charge) using pH control.

When the sorbent is charged it can be used to bind with positively charged compounds, such as the analytes in this assay, which would have most likely bound irreversibly to a strong cation exchanger. Using the WCX phase, the cationic analytes can then be desorbed from the sorbent by increasing the acidity of the elution phase to switch off (protonate the charged surface groups) the sorbent, which then causes the analytes to be eluted off.

Additional sensitivity can be gained with the use of the 30 mg format of the SOLA WCX cartridge. It provides greater sample loading capacity, which allows a greater volume of sample to be loaded onto the sorbent without analyte break-through. This in part allows this assay to reach extremely low levels of detection (50 pg/mL) for all the analytes, while demonstrating low matrix effects and exceptionally high recoveries.

Conclusion

This application note clearly demonstrates the excellent separation performance of the Accucore Polar Premium column and the excellent selectivity of the SOLA WCX 30 mg SPE phase for the quantitation of acetylcholinesterase inhibitors. Additionally, the extremely high precision and high accuracy demonstrate the robustness of this assay even at the low LOQ of 50 pg/mL due to the unique design of the SOLA product, which enables reproducible processing of biological samples. The power of the SOLA WCX SPE phase is highlighted with recoveries for the three analytes pyridostigmine, edrophonium, and neostigmine of 104.8%, 91.8%, and 91.2%, respectively, using a simple SPE clean-up. This SPE method was followed by a rapid 3.25 min chromatographic separation utilizing the separating power of the Accucore Polar Premium phase to separate the analytes.

Find out more at **thermofisher.com/solaspe**

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