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Application Note

# A Method for Choline and Acetylcholine Using Hydrophilic Interaction Chromatography (HILIC) and Mass Spectrometry

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## Abstract

In this application note, we have developed and optimized a stationary phase for HILIC that provides good retention of very polar basic analytes, including several active pharmaceutical ingredients and their metabolites and/or impurities.

## Benefits

The Atlantis HILIC Silica Column offers a unique selectivity that retains and resolves Ch and ACh

## Introduction

Hydrophilic interaction chromatography (HILIC) is a useful technique for the retention of polar analytes that offers a difference in selectivity compared to traditional reversed-phase (RP) chromatography. The highly volatile organic mobile phases used in HILIC provide increased electrospray ionization-mass spectrometry (ESI-MS) sensitivity. An Atlantis HILIC Silica Column was used to develop a quantitative HILIC LC-MS method for the analysis of acetylcholine and choline. A limit of detection of 0.1 ng/mL was achieved for acetylcholine on a single quadrupole mass spectrometer. Finally, a simplified solid phase extraction (SPE) procedure using the new Oasis WCX sorbent is described. The evaporation and reconstitution steps can be eliminated with the direct injection of extracted organic fractions from SPE devices. This leads to lower method variability and higher throughput.

We have developed and optimized a stationary phase for HILIC that provides good retention of very polar basic analytes, including several active pharmaceutical ingredients and their metabolites and/or impurities. HILIC utilizes mobile phases that are highly volatile (>80% organic) which is ideal for efficient desolvation and compound ionization, in effect enhancing signal response in mass spectrometry compared to traditional RP chromatographic methods. This enhancement effect allows for low limits of detection. An Atlantis HILIC Silica Column was used to develop a sensitive, quantitative HILIC-based LC-MS method for the analysis of the quaternary amines, acetylcholine and choline. Choline is important for phospholipid formation, normal membrane function, and the synthesis of neurotransmitters such as acetylcholine. Because of the high organic mobile phase starting conditions, samples in biological matrices cleaned-up by SPE can be directly analyzed by LC-MS without solvent evaporation and reconstitution. For this application, the new mixed-mode, weak cation exchange sorbent, Oasis WCX was utilized. This sorbent provides a selective clean-up as well as a mass spectrometry friendly elution solvent that can be directly injected onto the HILIC column.

## Experimental

### Chromatographic Conditions

| | |
|---|---|
| Column: | Atlantis HILIC Silica 2.1 x 50 mm, 3 µm |
| Mobile phase A: | $H_2O$ |

| | |
|---|---|
| Mobile phase B: | ACN |
| Mobile phase C: | 200 mM NH4COOH, pH 3.0 |
| Flow rate: | 0.3 mL/min |
| Isocratic mobile phase: | 9% A; 86% B; 5% C |
| Injection volume: | 20 µL |
| Sample diluent: | 60:40 IPA:ACN with 0.2% FA |
| Sample concentration: | 0.1 ng/mL acetylcholine (ACh); 1.0 ng/mL choline (Ch); 5 ng/mL choline-$d_9$ (Ch-$d_9$) |
| Temperature: | Ambient |
| Instrument: | Waters Alliance HT System, 2795 Separations Module with Waters ZQ |
| SDMS: | Empower Build 1154 |

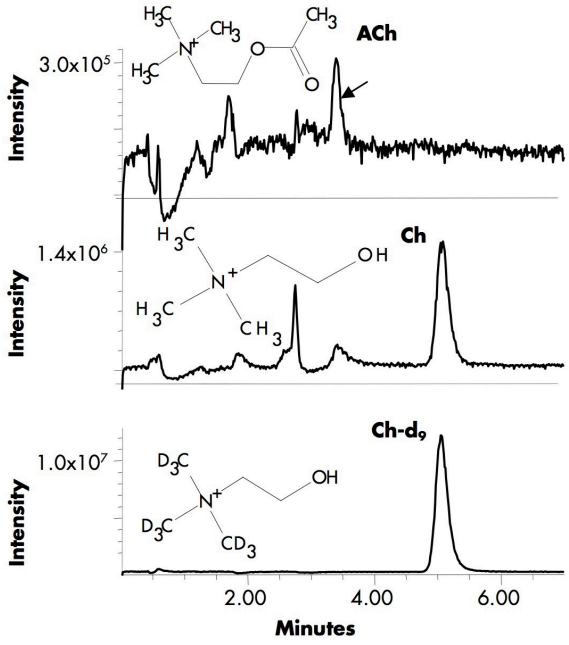
## MS Conditions

| | |
|---|---|
| Capillary (kV): | 1 |
| Cone (V): | 15 (ACh): 30 (Ch, Ch-$d_9$) |
| Extractor: | 3 V |
| RF lens: | 0.3 V |

| | |
|---|---|
| Source temp. (°C): | 150 |
| Desolvation temp. (°C): | 350 |
| Cone gas flow (L/Hr): | 50 |
| Desolvation gas flow (L/Hr): | 700 |
| SIR *m/z*: | 146.2 (ACh), 103.9 (Ch), 113.1 (Ch-$d_9$) |

## Results and Discussion

A HILIC-ESI-MS method was developed that offers a unique selectivity that retains and resolves Ch and ACh. This highly sensitive method results in a limit of detection of 0.1 ng/mL for ACh on a single quadrupole mass spectrometer. Analyte concentrations were analyzed over the working range of 0.1–100 ng/mL to determine the limit of detection (LOD) and limit of quantitation (LOQ). Ch-$d_9$ was used as the internal standard and held at a constant 5 ng/mL. Data from the calibration curves generated are listed in Table 1.




| Approximate Limits of Detection (Analyte Dependant) | | |
|---|---|---|
| | Reversed-phase | **HILIC** |
| **Single Quadrupole MS** (SIR) mode ES+ | 10 ng/mL | **1 ng/mL** |
| **Triple Quadrupole MS** (MRM) mode ES+ | 1 ng/mL | **0.1 ng/mL** **[100 fg/µL]** |

*HILIC-ESI-MS will achieve at least 10x higher sensitivity with high organic mobile phases than typical RP-ESI-MS with high aqueous mobile phases.*

## Table 1

Precision and Accuracy of Calibration Standards (N = 6)

| *ACh (ng/mL)* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Nominal concentration | 0.10 | 0.25 | 0.50 | 1.00 | 2.50 | 5.00 | 10.00 | 25.00 | 50.00 | 100.00 |
| Calculated mean conc. | 0.11 | 0.24 | 0.47 | 1.15 | 2.41 | 4.71 | 9.74 | 24.76 | 48.63 | 102.13 |
| RSD (%) | 4.88 | 4.59 | 2.93 | 3.26 | 1.98 | 1.93 | 2.45 | 1.66 | 1.82 | 1.05 |
| RE (%) | 8.65 | -5.06 | -5.32 | 15.38 | -3.61 | -5.88 | -2.60 | -0.95 | -2.74 | 2.13 |

| *Ch (ng/mL)* | | | | | | | |
|---|---|---|---|---|---|---|---|
| Nominal concentration | 1.00 | 2.50 | 5.00 | 10.00 | 25.00 | 50.00 | 100.00 |
| Calculated mean conc. | 1.37 | 2.43 | 4.17 | 8.29 | 23.47 | 49.50 | 104.24 |
| RSD (%) | 0.79 | 2.22 | 1.44 | 0.97 | 0.53 | 0.63 | 1.33 |
| RE (%) | 36.50 | -2.63 | -16.55 | -17.0 | -6.13 | -1.00 | -4.24 |

The data was best fit using a weighted (1/x) linear regression yielding correlation coefficients of 0.995 and 0.999 for Ch and ACh, respectively. Due to a limitation of the linear range for Ch, the LOD and LOQ were determined to be 1.0 ng/mL and 2.5 ng/mL, respectively. Based on a signal-to-noise ratio of 3:1, the LOD for ACh was 0.1 ng/mL. Based on a signal-to-noise ratio of 10:1, the LOQ for ACh was 0.25 ng/mL.

## Simplified Sample Preparation Procedure

Traditional SPE methods often contain an elution step that uses a large concentration (>75%) of an organic solvent, which often results in the time-consuming evaporation and reconstitution steps. The high organic SPE eluent is compatible with the high organic mobile phase conditions that HILIC requires, thus eliminating the need to evaporate and reconstitute the SPE eluent. For the most selective SPE clean-up of bases, a cation exchange sorbent is recommended. In this case, because the analytes are always charged (*i.e.* quaternary amines), the new Oasis WCX (Weak Cation eXchange) material is the best choice for SPE.

## Sample Preparation Procedure

A solution spiked with a concentration of 2.5 ng/mL was prepared. A volume of 25 µL of a mixture of ACh, Ch and Ch-$d_9$ (500 ng/mL each) in water was added to 4875 µL of Artificial Cerebral Spinal Fluid (aCSF). A volume of 100 µL of ammonium hydroxide was then added to basify the spiked solution at a final concentration of 2% (v/v). An aCSF blank was prepared by adding 100 µL of ammonium hydroxide to 4900 µL of aCSF to yield a final concentration of 2% (v/v) ammonium hydroxide.

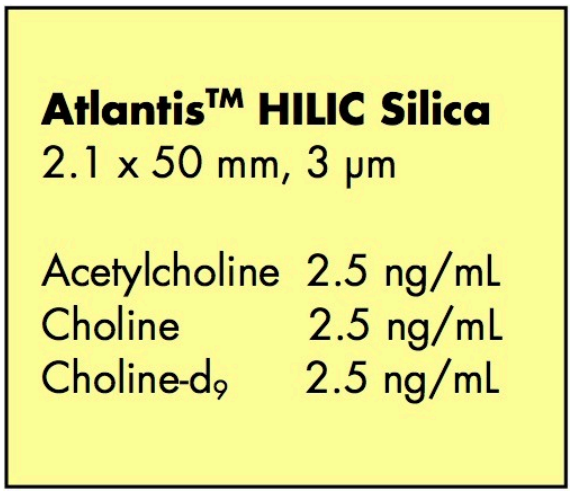
Solid phase extraction was performed using a 96-well µElution plate containing 2 mg/well Oasis WCX. The sorbent was conditioned with 200 µL of MILLI-Q water. The spiked solution was loaded onto the sorbent in a volume of 40 µL. A wash step of 100 µL of MILLI-Q water was performed followed by a second wash containing

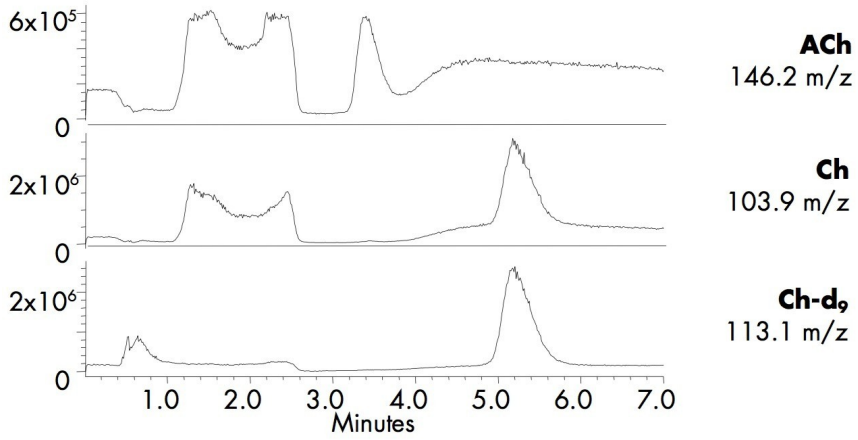
100 µL of methanol. Finally the analytes of interest were then eluted with 40 µL of 5% (v/v) formic acid in isopropanol-acetonitrile (60:40 v/v). This elution solvent is weaker than the mobile phase, *i.e.* 10 mM ammonium formate in acetonitrile:water (86:14), and was directly injected onto the HILIC column. The experiment was conducted with six replicates. To calculate the absolute recovery, 270 µL of an extracted aCSF blank was pooled from 12 wells and spiked with 30 µL of a 25 ng/mL mixture of ACh, Ch and Ch-$d_9$ in water to yield a final concentration of 2.5 ng/mL.Recovery results are reported in Table 2.

## Table 2

SPE Extraction Recoveries of ACh, Ch and Ch-$d_9$ (N=6)

| | *Ch* | *ACh* | *Ch-$d_9$* |
|---|---|---|---|
| Nominal Concentration (ng/mL) | 2.50 | 2.50 | 2.50 |
| Recovery (%) | 101 | 100 | 98 |






## Conclusion

The Atlantis HILIC Silica Column offers a unique selectivity that retains and resolves Ch and ACh. This unique selectivity led to the development of a sensitive HILIC-ESI-MS method which allows for detection levels on a single quadrupole mass spectrometer that were once only obtainable on a tandem MS system. The new Oasis WCX µElution Plate offers the most selective SPE clean-up for strong bases and low elution volumes and when coupled with direct injection onto the Atlantis HILIC Silica Column, eliminates the need for evaporation and reconstitution.

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