

Development of Assays to Determine Cholinergic Agents and Related Substances by Ion Chromatography

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INTRODUCTION

Acetylcholine and structurally similar quaternary ammonium compounds are potent neurotransmitters that can affect both the parasympathetic and sympathetic nervous systems. Examples of diverse indications for acetylcholine-like agents are carbachol, used primarily for ophthalmic applications; bethanechol used to treat urinary retention; and methacholine used to assess bronchial asthma. When administered externally, changes in the concentration of these agents and their potential decomposition and synthetic impurity products can change the desired physiological response.

In this study, the authors describe the assay of selected cholinergic agents in model physiological saline solutions using a Reagent-Free™ ion chromatography system. This system uses deionized water, coupled with electrolytically generated methanesulfonic acid, to generate the eluent. Target analytes are separated on a Dionex IonPac® CS17 cation-exchange column and measured at high sensitivity by suppressed conductivity detection. Surrogate samples are prepared by spiking target analytes into model matrices and diluting with deionized water.

The results for the following cholinergic agents and their potential hydrolysis/synthesis impurities in model matrices are presented: carbachol and choline in an ophthalmic solution matrix, bethanechol and 2-hydroxypropyltrimethylammonium (β -methylcholine) also in an ophthalmic solution matrix, and methacholine, acetylcholine, β -methylcholine, and acetate (with anion IC) in physiological saline. This presentation shows the development of these methods and reviews the results, which include method detection limit, linear calibration range, spike recovery, retention time precision, and peak area precision determinations. The results show that IC is an accurate and reproducible technique to determine cholinergic agents and their related compounds in high-ionic strength samples.

EQUIPMENT

The study was performed using a Dionex ICS-2100 RFIC™ system, an integrated ion chromatography system consisting of an eluent generator, pump with in-line vacuum degas, column heater, AS autosampler, and Chromeleon® Chromatography Data System (CDS) software.

Standards were prepared using USP reference material (P/N 1092009 for carbachol chloride, P/N 1071009 for bethanechol chloride, P/N 1396364 for methacholine chloride, and P/N 1008501 for acetylcholine chloride). Base hydrolysis with 0.1 N NaOH was used to prepare choline standards from carbachol and to prepare β -methylcholine standards from either bethanechol or methacholine. Over-the-counter eye care solutions were spiked with carbachol and bethanechol. Methacholine was spiked into physiological saline with or without phenol preservative. Figure 1 shows the structures for the six cholinergic agents determined in this study. For more extensive details of the method, refer to Dionex AN 194 and AN 249.^{1,2}

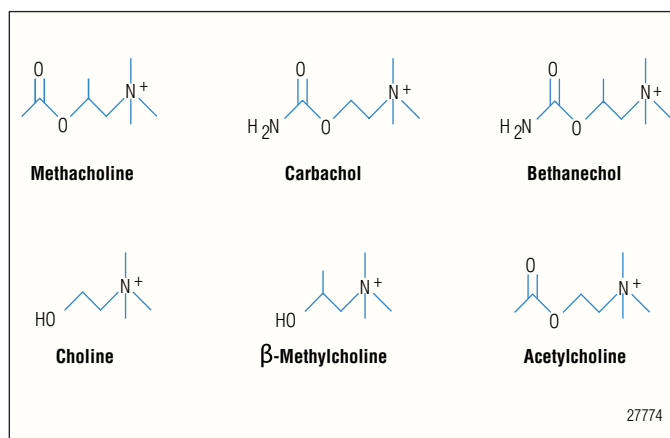


Figure 1. Chemical structures of choline and related compounds determined in this study.

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EXPERIMENTAL CONDITIONS

Cations

Columns: IonPac CS17 4 mm Analytical 4 × 250 mm
IonPac CG17 4 mm Guard 4 × 50 mm
Eluent: 5 mM Methanesulfonic acid
Eluent Source: EluGen II MSA
Flow Rate: 1.0 mL/min
Temperature: 30 °C
Injection Vol.: 25 µL
Detection: Suppressed conductivity, CSRS® 300 4 mm recycle mode, 20 mA

Anions

Columns: IonPac AS22 4 mm Analytical 4 × 250 mm
IonPac AG22 4 mm Guard 4 × 50 mm
Eluent: 4.5 mM Sodium carbonate/1.4 mM sodium bicarbonate. Manually prepared
Flow Rate: 1.2 mL/min
Temperature: 30 °C
Injection Vol.: 25 µL
Detection: Suppressed conductivity, ASRS® 300 4 mm recycle mode, 31 mA

RESULTS AND DISCUSSION

Chromatography

System suitability was determined for the separation of parent cholinergic agents and their possible choline-containing impurities. Figure 2 shows the separation of β-methylcholine, acetylcholine, and methacholine in the presence of commonly occurring cations. All components were well separated.

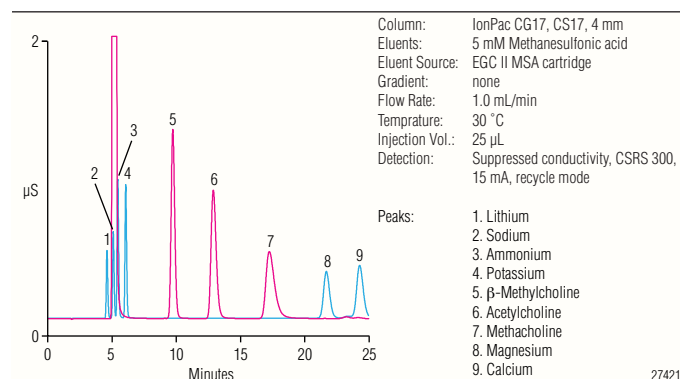


Figure 2. Overlay of β-methylcholine, acetylcholine, and methacholine with a mixed-cation standard.

Figure 3 shows a chromatogram of choline, carbachol, and bethanechol standards along with several commonly occurring cations. All the compounds were well separated from each other.

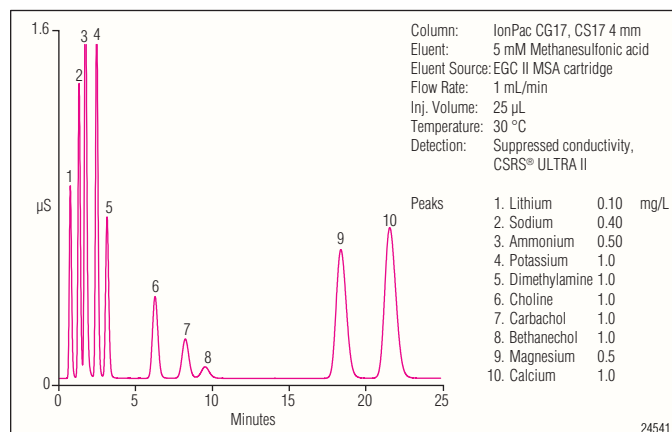


Figure 3. Separation of carbachol, choline, and bethanechol with a mixed cation standard.

Figure 4 shows the separation of bethanechol from 2-HPTA (β-methylcholine) during the decomposition of bethanechol in alkaline solution.

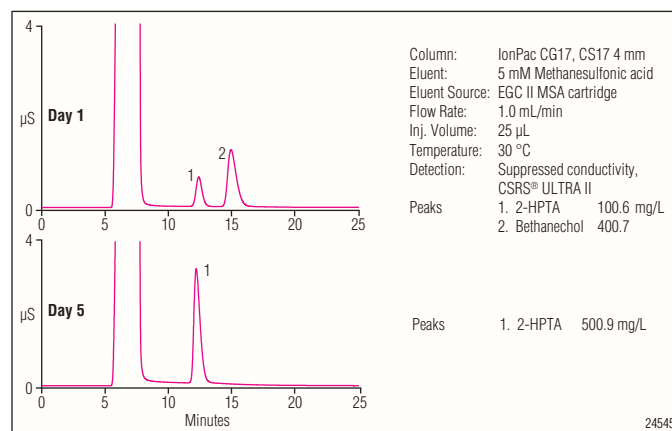


Figure 4. Conversion of bethanechol to 2-HPTA (β-methylcholine) in the presence of 0.1 N NaOH.

LINEARITY

Table 1 summarizes the calibration results for the parent cholinergic agents and their potential impurities in deionized water.

Table 1. Calibration Results for Methacholine, Carbachol, Bethanechol, and their Potential Impurities					
Analyte	Agent	Range (mg/L)	Correlation Coefficient (r ²)	Offset (µS·min)	Slope
Methacholine	Methacholine	5–50	0.99995	-0.032	0.036
Acetylcholine		0.1–100	0.99991	-0.016	0.040
β-Methylcholine		0.1–100	0.99997	-0.017	0.050
Acetate		0.1–50	0.99983	0.001	0.015
Carbachol	Carbachol	0.02–1000	0.99998	-0.036	0.085
Choline		0.02–500	0.99999	-0.011	0.036
Bethanechol	Bethanechol	0.02–1000	0.00028	-0.007	0.036
β-Methylcholine		0.02–500	0.99997	-0.011	0.028

MDL ESTIMATES FOR POTENTIAL IMPURITIES

Assay development for the parent cholinergic agent does not require estimating the minimum detection limit (MDL) for the parent compound but MDLs of impurities are important for developing related substance assays. Table 2 summarizes MDL estimates for acetylcholine, β-methylcholine, and acetate potential impurities in methacholine, choline potential impurity in carbachol, and β-methylcholine potential impurity in bethanechol.

Table 2. Estimated Method Detection Limits for Potential Impurities in Methacholine, Carbachol, or Bethanechol		
Analyte	Agent	Calculated MDL (µg/L)
Acetylcholine	Methacholine	8
β-Methylcholine		5
Acetate		75
Choline	Carbachol	1
β-Methylcholine	Bethanechol	5

SPIKE RECOVERY

Recoveries of methacholine and its potential impurities were studied at multiple concentrations in physiological saline with or without phenol preservative. Simulated matrices were diluted 1000-fold for methacholine assay and 100-fold for potential impurities analysis. Table 3 summarizes these results.

Carbachol was spiked into two over-the-counter ophthalmic solutions. After a 1000-fold dilution with DI water, carbachol recovery was evaluated. Table 4 summarizes the recovery results.

Table 3. Recoveries of Methacholine and Possible Impurities in Simulated Matrices

Analyte	Matrix ^a	Spiking Level (mg/L)	Molar % of Methacholine ^b	Recovery (%)	Precision (RSD)
Methacholine	9 mg/L NaCl	10	—	97.2	0.4
		25	—	98.5	0.3
		45	—	99.2	0.2
	9 mg/L NaCl 4 mg/L phenol	10	—	97.2	0.4
		25	—	98.1	0.1
		45	—	99.9	0.2
Acetylcholine	250 mg/L methacholine 90 mg/L NaCl	2.5	1.1	95.0	0.4
		25	11	97.1	0.2
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	1.1	85.4	0.2
		25	11	97.2	0.2
β-Methylcholine*	250 mg/L methacholine 90 mg/L NaCl	2.5	1.4	88.7	0.9
		25	14	98.3	0.1
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	1.4	84.7	0.3
		25	14	96.7	0.2
Acetate*	250 mg/L methacholine 90 mg/L NaCl	2.5	2.7	88.7	0.9
		25	27	98.1	0.2
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	2.7	92.4	0.8
		25	27	99.8	0.1

Table 4. Recovery of Choline from Two Eye Care Products

Matrix ^a	Spiking Level (mg/L)	Recovery (%)	Precision (RSD)
Lens solution	0.5	96	0.77
Saline solution	0.5	98	0.67

^a Diluted 1000-fold with DI water

PRECISION

Table 5 summarizes the results of a five-day study of retention time and peak area reproducibilities for methacholine and possible impurities. Eight replicates for each target analyte in each of the two sample matrices were injected daily.

Table 6 summarizes a short-term experiment in which between-day reproducibility was measured by injecting five replicates each day for six days.

Table 5. Retention Time and Peak Area Reproducibilities for Methacholine and Possible Impurities in Simulated Matrices			
Analyte (conc.)	Matrix ^a	Average RT, min (RSD)	Average Peak Area $\mu\text{S}\cdot\text{min}$ (RSD)
Methacholine (25 mg/L)	9 mg/L NaCl	17.019 (0.040)	0.8190 (0.31)
	9 mg/L NaCl 4 mg/L phenol	17.013 (0.052)	0.8359 (0.40)
Acetylcholine (10 mg/L)	250 mg/L methacholine 90 mg/L NaCl	12.772 (0.060)	0.3647 (0.45)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	12.780 (0.047)	0.3634 (0.42)
β -Methylcholine (10 mg/L)	250 mg/L methacholine 90 mg/L NaCl	9.642 (0.091)	0.4938 (0.75)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	9.638 (0.10)	0.4960 (0.70)
Acetate (2.5 mg/L)	250 mg/L methacholine 90 mg/L NaCl	3.249 (0.34)	0.0438 (1.1)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	3.248 (0.28)	0.0441 (0.85)

Table 6. Retention Time and Peak Area Reproducibilities for Choline in Two Eye Care Products			
Matrix ^a	Spiking Level (mg/L)	Retention Time RSD	Peak Area RSD
Lens solution	5	0.10	0.88
Saline solution	5	0.04	0.89

^a Samples diluted 1000-fold with DI water

CONCLUSION

- Methacholine, carbachol, and bethanachol can be quantified in high-ionic strength samples up to four orders of magnitude using a linear calibration model having r^2 values ≥ 0.9999 .
- Potential impurities in the three parent cholinergic agents can be quantified in high-ionic strength samples over 3–4 orders of magnitude using a linear calibration model having r^2 values ≥ 0.9998 .
- Estimated lower limit of detection for the choline-related potential impurities are calculated to be in the single-digit $\mu\text{g/L}$ range. Acetate lower limit of detection is $75 \mu\text{g/L}$.
- Method accuracy range from 96–100% for the parent cholinergic agents and 85–100% for potential impurities.
- Separations are reproducible with retention time RSDs that range from 0.04–0.10% for choline-related compounds and 0.3% for acetate while peak area RSDs that range from 0.3–1.1%. Lower acetate retention time reproducibility may be due to use of manually prepared eluent compared to the electrolytically produced eluent for the cation determinations.
- All parent cholinergic agents are well separated from their respective potential impurities and from commonly occurring cations. Analysis time was 25 min for choline-related compounds.

REFERENCES

1. Dionex Corporation. *Determination of Carbachol in Ophthalmic Solutions Using a Reagent-Free Ion Chromatography System*. Application Note 194, LPN 1967, 2008, Sunnyvale, CA.
2. Dionex Corporation. *Determination of Methacholine Chloride and Potential Impurities Using a Reagent-Free Ion Chromatography System*. Application Note 249, LPN 2517, 2010, Sunnyvale, CA.

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