DIONEX

Application Note 194

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Determination of Carbachol In Ophthalmic Solutions Using a Reagent-Free Ion Chromatography System

INTRODUCTION

Carbachol is a choline ester and a positively charged quaternary ammonium compound used primarily for ophthalmic applications, such as solutions used for glaucoma treatment or ophthalmic surgery. Carbachol is a potent cholinergic agent which constricts the iris and ciliary body resulting in reduction of intraocular pressure in patients with glaucoma.[1] The exact mechanism by which carbachol lowers intraocular pressure is not precisely known; however it is believed to increase the amount of fluid drained from the eye.

Diminished concentrations of carbachol in an ophthalmic formulation may prevent effective reduction of intraocular pressure, which may have deleterious effects such as iris prolapse. Analytical methods are therefore needed to ensure that concentrations in these solutions remain at therapeutically active levels. The current USP monograph (USP 29-NF 24) describes a colorimetric method for the determination of carbachol in ophthalmic solutions.[2] Colorimetric methods can be both time- and labor-intensive, and yield significant measurement errors.

Choline is a member of the B vitamin group and the parent member of a class of drugs referred to as cholinergic. Carbachol and bethanechol are two clinically useful choline derivatives.[3] In alkaline solutions, carbachol degrades to choline. Therefore, a method to selectively detect carbachol and choline is required.

Prior Applications describe methods for detection of choline and other analytes related to carbachol. Dionex Application Note 124 (AN 124) describes the use of an IonPac® CS12A column for the determination of free and bound choline from dried milk in infant formula.[4] In the method described here, optimized conditions for carbachol analysis were used to determine choline linearity, method detection limits (MDL), and separation from carbachol in lens and saline solutions.

Bethanechol chloride is a quaternary ammonium compound that is structurally and pharmacologically related to carbachol.[5] AN 148 reports a Reagent-Free™ Ion Chromatography (RFIC™) method for the determination of bethanechol chloride. Method parameters used for determining carbachol can also be used for bethanechol analysis, as shown in this Application Note. Linearity, MDL, and potential interferences with the breakdown product of bethanechol, 2-hydroxypropyltrimethylammonium chloride (2-HPTA) were also determined.

Here, a simple RFIC method is described for determination of carbachol, bethanechol, and choline in 25 min with a Dionex IonPac CS17 column. Methanesulfonic acid (MSA) eluent is delivered isocratically by an Eluent Generator (EG). Use of an EG eliminates eluent preparation errors and helps ensure retention time reproducibility. The sensitivity of suppressed conductivity detection allows carbachol determination in ophthalmic solutions with only a simple sample dilution.

EQUIPMENT

ICS-2000 (Dionex P/N 061098)

AS Autosampler

Chromeleon® 6.8 SP2 Chromatography Workstation

CONSUMABLES AND REAGENTS

CR-CTC II (Dionex P/N 066202)

CSRS® ULTRA II 4 mm (Dionex P/N 061563)

EluGen® II MSA Cartridge (Dionex P/N 058902)

Carbachol Chloride (USP reference standard P/N 1092009)

Bethanechol Chloride (USP reference standard P/N 1071009)

Six Cation Standard II (Dionex P/N 046070)

Dimethylamine (Fluka P/N 38960)

Alcon OPTI-FREE® RepleniSH™ Multipurpose Disinfecting Lens Solution

Bausch & Lomb Gentle Sensitive Eyes® Plus Saline Solution

Type I reagent-grade distilled water or deionized water with a specific resistance of 17.8 MΩ -cm or greater, filtered through a 0.2 µm filter immediately before use.

CONDITIONS

Columns:	IonPac CG17 4 mm 4 x 50 mm (Dionex PN 060560) IonPac CS17 4 mm 4 x 250 mm (Dionex PN 060557)
Eluent:	5 mM Methanesulfonic Acid
Flow Rate:	1.0 mL/min
Temperature:	30 °C
Injection Volume:	25 µL
Detection:	Suppressed conductivity, CSRS ULTRA 4mm (P/N 053948) recycle mode
Power setting:	20 mA
Background Conductivity:	< 1 µS
Noise:	< 0.5 nS/min
Backpressure:	2300 psi
Run Time:	25 min

ELUENT SOLUTION

5 mM MSA eluent is generated on-line using an EG Eluent Generator with an MSA EluGen cartridge. Fill the eluent reservoir with reagent water and maintain an inert helium atmosphere of 3-5 psi in the eluent reservoir. Chromeleon software tracks the amount of MSA used and calculates the remaining lifetime. Replace the MSA cartridge when the remaining lifetime drops below 10%.

Alternately, manually prepared MSA may be used. First prepare a 1.0 N stock solution by adding 96.10 g of MSA to a 1 L volumetric flask containing approximately 500 mL of deionized water. Bring to volume with deionized water, and mix thoroughly. Prepare 5 mM MSA by diluting 5 mL of the 1 N MSA stock solution to 1 L with deionized water. Degas the eluent and store in a plastic container.

STOCK STANDARD SOLUTIONS

1000 mg/L Carbachol Solution

Dissolve 0.1762 g of carbachol chloride in approximately 75 mL of reagent water and dilute to 100 mL in a volumetric flask. Store the stock solution in a high-density polyethylene or polypropylene bottle at 4 °C.

1000 mg/L Choline Solution

Weigh 0.0881g of carbachol chloride into a 125 mL plastic bottle, add 50 mL of 0.1N NaOH, sonicate to dissolve, and mix. Allow five days for the carbachol to completely hydrolyze to choline.

1000 mg/L Bethanechol Solution

Dissolve 0.1 g of bethanechol chloride in approximately 75 mL of reagent water and dilute to 100 mL in a volumetric flask. Store the stock solution in high-density polyethylene or polypropylene bottle at 4 °C.

1000 mg/L 2-Hydroxypropyltrimethylammonium (2-HPTA) Solution

HPTA was prepared as directed in AN 148. Weigh 0.050g of bethanechol chloride into a 125 ml plastic bottle, add 50 mL 0.1N NaOH, sonicate to dissolve, and mix. Allow five days for the bethanechol to completely hydrolyze to 2-HPTA chloride.

1000 mg/L Dimethylamine Solution

Dissolve 0.1 g of dimethylamine in approximately 75 mL of reagent water and bring to volume in a 100 mL volumetric flask. Store the stock solution in a high-density polyethylene or polypropylene bottle at 4 °C.

WORKING STANDARD SOLUTIONS

To prepare working standards, use a calibrated pipet to deliver the appropriate volume of the 1000 mg/L stock standard into a volumetric flask and bring to volume with reagent grade water. For method linearity studies, the following standards of bethanechol, choline, carbachol,

and 2-HPTA were used: 1000, 500, 200, 100, 50, 25, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 mg/L. The exceptions were the linearity studies on 2-HPTA and choline, with maximum concentrations of 500 mg/L.

To prepare mixed standards containing carbachol and other compounds, combine appropriate volumes of the carbachol stock standard with the Cation Standard II, or single-component standards, in a volumetric flask and bring to volume with reagent water.

INTERFERENCE STUDIES

To confirm no other compounds interfere with carbachol determinations using this method, a mixed standard was injected containing carbachol (1 mg/L) along with lithium (0.1 mg/L), sodium (0.4 mg/L), ammonium (0.5 mg/L), potassium (1 mg/L), magnesium (0.5 mg/L), calcium (1 mg/L), choline (1 mg/L), bethanechol (1 mg/L), and dimethylamine (1 mg/L).

SAMPLES

Alcon OPTI-FREE RepleniSH Multipurpose Disinfecting Lens Solution and Bausch & Lomb Gentle Sensitive Eyes Plus Saline Solution were each diluted 1:1000 with reagent water and spiked with the desired amount of carbachol for linearity and (MDL) determinations as well as recovery and precision studies.

SYSTEM PREPARATION AND SETUP

Verify that the pump flow rate is within specifications and recalibrate if necessary. (The pump should deliver liquid at ± 0.5% of the specified volume against a constant backpressure of 2300 psi.) Verify that the conductivity cell constant is within specifications and recalibrate if necessary. Consult the pump or detector manuals for procedural details.

Install the EG, and condition the EluGen II MSA Cartridge as directed in the manual by running a gradient from 1 to 60 mM MSA in 20 min, then 60 mM for 40 min at 1 mL/min. (For instructions on installation and use, see the ICS-2000 IC system installation instructions, Document No. 031857.)

Install and configure the autosampler. Use a calibrated sample loop in "full loop" mode to obtain the best accuracy and precision. Note: If making partial loop injections, program a sample volume that is less than half the volume of the installed sample loop, with a cut volume of 8 µL. This injection procedure should provide peak area precision of < 1% RSD.

Install a 1-mL sample syringe and set the syringe speed to 3. Enter the correct sample loop size and sample syringe volume in the AS plumbing configuration screen. Refer to the ICS-2000 Ion Chromatography System Installation Instructions, (Document No. 031857) for details.

Install an IonPac CG17 4 x 50 mm guard column and an IonPac CS17 4 x 250 mm analytical column. Confirm that the system pressure displayed by the pump is at least 2300 psi when 5 mM MSA is delivered at 1.0 mL/min. This allows the degas assembly to effectively remove electrolysis gas from the eluent. If necessary, install backpressure coils supplied with the EG ship kit to adjust the system pressure to between 2300 and 2800 psi. Because system pressure can rise over time, it may be necessary to trim the backpressure coil to maintain system pressure under 3000 psi. Do not exceed 3000 psi or the degas assembly tubing may rupture.

Prepare the CSRS-ULTRA 4 mm suppressor for use by hydrating the eluent chamber. Pump approximately 5 mL reagent water through the Regen In port. Next, pump approximately 5 mL reagent water through the Eluent In port. Allow approximately 20 min to fully hydrate the suppressor screens and membranes. Install the CSRS-ULTRA in Recycle Mode, following the installation and troubleshooting instructions for the CSRS-ULTRA, (Document No. 031370).

Equilibrate the column with 6 mM MSA eluent for 60 min, and analyze a system blank of reagent water. A well-equilibrated system should have a background conductivity of approximately 1 µS, and peak-to-peak noise should be < 0.5 nS/min.

Make a 25 µL full injection of a 1:1000 dilution of the six cation standard along with 1 mg/L carbachol. None of the peaks in the standard should coelute with carbachol. Once the column is equilibrated, duplicate injections of the standard should produce identical or nearly identical retention times for all analytes.

Peak area precision and accuracy depend on the performance of the autosampler. The water in the flush reservoir should be replaced daily, and the sample syringe and tubing should be regularly inspected for bubbles. If bubbles are observed, they should be removed by purging as outlined in the autosampler manual. The injection mode used also affects precision and accuracy; the most accurate way to make an injection is by using a calibrated sample loop, in "full loop" injection mode.

RESULTS AND DISCUSSION

Chromatography and Interference Studies

In order to determine the system suitability for the analysis of carbachol in the presence of commonly occurring cations, the compound was analyzed in the presence of lithium, sodium, ammonium, potassium, magnesium, calcium, choline, bethanechol, and dimethylamine. Figure 1 shows a chromatogram of a 1 mg/L carbachol standard along with several commonly occurring cations, and compounds that may potentially interfere with the analysis of carbachol. The retention times of lithium, sodium, ammonium, potassium, dimethylamine, choline, carbachol, bethanechol, magnesium, and calcium were 4.01, 4.50, 4.87, 5.51, 6.10, 8.81, 10.53, 11.66, 19.35 and 22.13, minutes, respectively. Thus, all the compounds are well separated from carbachol, and do not interfere with its determination. Figure 2 shows the conversion of carbachol to choline in the presence of NaOH, on Day 1 and Day 5 of exposure to 0.1N NaOH.

Determination of Linearity for Carbachol and Choline

Prior to evaluation of carbachol in samples, a calibration using different concentrations of carbachol was performed with the standards prepared in reagent grade water. Table 1 summarizes the data for a typical calibration curve obtained by injecting calibration standards at 1000, 500, 200, 100, 50, 25, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 mg/L of carbachol. Table 1 also summarizes the calibration data for choline using the same calibration standards with the exception of the 1000 mg/L standard. Calibration for both compounds was linear over four orders of magnitude, with a correlation coefficient of 0.9999 for carbachol and choline.

Table 1. Linear Range for Carbachol and Choline

Analyte	Range (mg/L)	r^2	Offset	Slope
Carbachol	0.02 – 1000	0.99998	-0.036	0.085
Choline	0.02 – 500	0.99999	-0.011	0.060

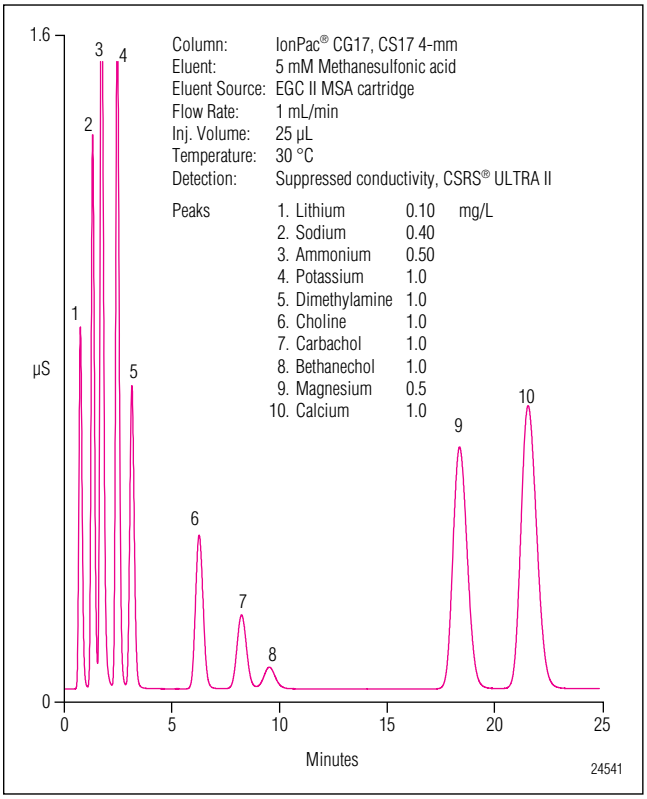

Figure 1. Separation of 1 mg/L carbachol, choline, and bethanechol with a mixed cation standard.

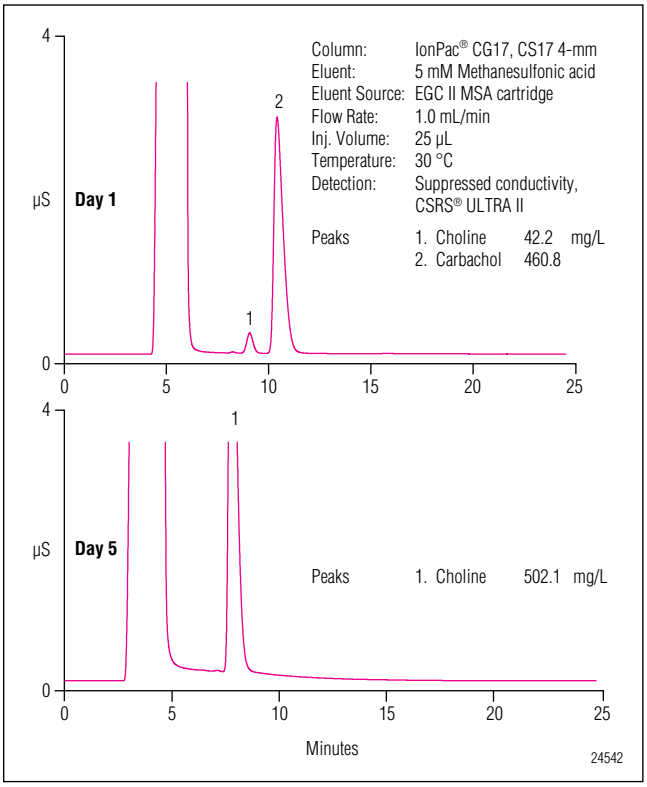

Figure 2. Conversion of carbachol to choline in the presence of 0.1 N NaOH.

Minimum Detection Limit (MDL) for Carbachol and Choline

The MDL is defined as the minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero. It is a measure of the precision of preparing and analyzing low-level samples according to the method parameters. The MDL of carbachol was determined by making seven injections of a low-level solution fortified with carbachol at a level yielding a signal/noise ratio of approximately 3–5 µS. The amount was determined using the calibration curve, and the MDL was calculated.

The MDL for carbachol in water was determined by making seven replicate injections of reagent water fortified with carbachol at 0.02 mg/L. Using this method, the calculated MDL for carbachol in water is 5 µg/L. The calculated MDL for choline obtained by the same method is 1 µg/L. Table 2 summarizes the data for the determination of the MDLs for carbachol and choline.

Table 2. Determination of Carbachol and Choline MDLs

Analyte	Range (mg/L)	MDL Standard (mg/L)	RSD	S/N	Calculated MDL (µg/L)
Carbachol	0.02 – 1000	0.02	0.12	5.61	5
Choline	0.02 – 500	0.01	0.04	2.96	1

** The MDLs were calculated as MDL = (t) x (SD), where t = Student's t value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom [t = 3.14] for seven replicates of the MDL Standard, and SD = standard deviation of the replicate analysis.*[5]

SAMPLE ANALYSIS

Carbachol is used in wash solutions for surgical procedures. As these solutions are not commercially available, this method was developed and tested using over-the-counter eyecare solutions, including Alcon Optifree RepleniSH Multi-Purpose Disinfecting Lens Solution and Bausch & Lomb Gentle Sensitive Eyes Plus Saline Solution spiked with carbachol. These solutions share similar properties to the wash solutions used during surgical procedures. Because they contain large amounts of sodium, sample dilution is necessary to prevent overloading of the column with the matrix ions. Overloading may cause the carbachol peak to appear shorter and broader, may reduce carbachol recovery, and may compromise integration reliability.

Figure 3 shows determination of 1 mg/L carbachol spiked into Alcon Lens Solution diluted 1:1000. Sodium was observed along with carbachol. The Bausch & Lomb saline solution was also diluted 1:1000 and spiked with carbachol. Figure 4 shows determination of 1 mg/L carbachol spiked into diluted Bausch & Lomb saline solution using optimized conditions. The solution contains sodium and potassium. Neither cation interferes with carbachol in either matrix.

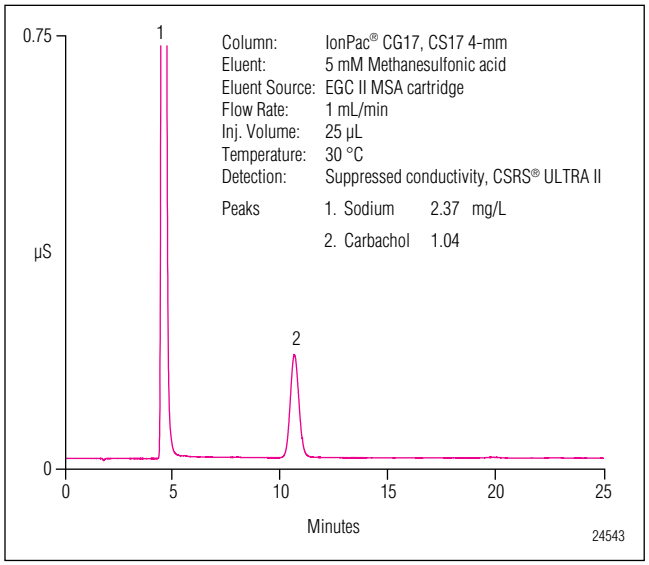

Figure 3. Alcon Lens Solution (diluted 1:1000) spiked with 1 mg/L of carbachol.

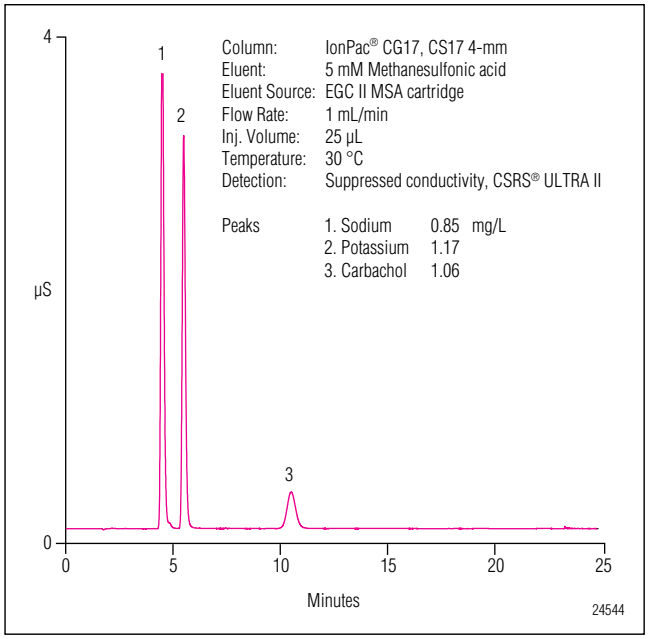

Figure 4. Saline solution (diluted 1:1000) spiked with 1 mg/L of carbachol.

Carbachol was spiked into the two matrices mentioned above, and precision, recovery, linearity, and MDL were evaluated. The results from the linearity and MDL studies for both matrices are summarized in Table 3. Precision and recovery data are shown in Table 4.

Table 3. Linear Range and Detection Limits of Carbachol in Two Over-the-Counter Ophthalmic Solutions

Matrix	Range (mg/L)	r2	MDL Standard (mg/L)	RSD	S/N	Calculated MDL (µg/L)
Alcon Lens Solution	0.01 – 500	0.99999	0.02	0.035	4.12	4
Bausch & Lomb Saline Solution	0.01 – 500	0.99995	0.01	0.11	13.5	3

** MDLs were calculated as MDL = (t) x (SD) where t = Student's t value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom [t = 3.14] for seven replicates of the MDL Standard, and SD = standard deviation of the replicate analysis.*[5]

Table 4. Recovery of Carbachol from Ophthalmic Samples

Matrix	Amount Added (mg/L)	Recovery	Precision (RSD)
Alcon Lens Solution	0.5	96	0.77
Bausch & Lomb Saline Solution	0.5	98	0.67

Short-term, between-day reproducibility was measured by injecting five replicates of a 5 mg/L standard each day for 6 days. The between-day the precision based on the retention time RSD was 0.043% with saline and 0.101% with lens solution. The high retention time reproducibility is a result of a continuous generation of high-purity eluent by the eluent generator, which provides an increased level of automation, decreased operator error, and greater precision as compared to manual preparation of mobile phases.

Determination of Bethanechol

As shown in Dionex AN 148, bethanechol undergoes hydrolysis to 2-HPTA in the presence of an alkaline solution, therefore 2-HPTA was also evaluated in this study. Different concentrations of bethanechol standards were prepared in reagent grade water and a calibration procedure was performed. Table 6 shows a typical calibration curve obtained by injecting standards at 1000, 500, 200, 100, 50, 25, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 mg/L of bethanechol. Table 6 also summarizes the calibration data for 2-HPTA using the same concentrations, with the exception of the 1000 mg/L standard. Calibration for both compounds was linear over four orders of magnitude, with a correlation coefficient of 0.9999 for bethanechol and 2-HPTA. Table 7 summarizes the data for the determination of MDL for those two compounds. Figure 5 shows the conversion of bethanechol to 2-HPTA in an alkaline solution.

Separation of choline from carbachol and bethanechol was also evaluated in two over-the-counter eye care products. Figure 6 shows separation of choline from carbachol and bethanechol when using lens solution as a matrix. The chromatogram demonstrates reliable separation, even in the presence of sample matrix cations.

Table 5. Reproducibility

Matrix	Concentration (mg/L)	RSD		
		Retention Time	Height	Area
Alcon Lens Solution	5	0.10	0.78	0.88
Bausch & Lomb Saline Solution	5	0.43	0.74	0.89

Table 6. Linear Range for Bethanechol and 2-HPTA

Analyte	Range (mg/L)	r2	Offset	Slope
Bethanechol	0.02 – 1000	0.99928	-0.007	0.036
2-HPTA	0.02 – 500	0.99997	-0.011	0.028

Table 7. Determination of MDL for Bethanechol and 2-HPTA

Analyte	Range (mg/L)	MDL Standard (mg/L)	RSD	S/N	Calculated MDL (µg/L)
Bethanechol	0.02 – 1000	0.05	0.05	2.8	2
2-HPTA	0.02 – 500	0.05	0.12	3.3	5

** The MDLs were calculated as MDL = (t) x (SD) where t = Student's t value for a 99% confidence level and a standard deviation estimate with n – 1 degrees of freedom [t = 3.14] for seven replicates of the MDL Standard, and SD = standard deviation of the replicate analysis.*[5]

PRECAUTIONS

Carbachol is hazardous to humans and to the environment. It can be toxic if swallowed and harmful if inhaled. It may cause skin irritation, and may be harmful if absorbed through the skin. It may cause irritation of the eyes as well as the upper respiratory tract and mucous membranes. To dispose of this material, contact a licensed waste disposal service.

Bethanechol chloride and choline are harmful if inhaled, swallowed, or absorbed through the skin. These materials may cause serious damage to the eyes; wear protective gloves and clean body-covering clothing, chemical safety goggles, and work in a well-ventilated area. These materials should be disposed of in accordance with the appropriate federal, state and local regulations.

Strongly retained compounds can accumulate on the column and degrade its performance. Signs of a fouled column include loss of capacity, loss of resolution, shortened retention times, higher noise and background, spurious peaks, and peak tailing. When cleaning an analytical and guard column in series, ensure that the guard column is placed after the analytical column in the flow path. Flush the columns for 15 min with 10 mM HCl at a flow rate of 1.0 mL/min, followed by a 1M HCl flush for 60 min to help remove contaminants. (For more information on column troubleshooting and cleanup, see the installation instructions and troubleshooting guide for the IonPac CS17 analytical Column, Document No. 031877.)

Some samples contain particulates that may plug the column and increase backpressure. Use a guard column to protect the analytical column. Inspect the column bed supports for discoloration and change if discolored. Replace the guard column if a sample causes a sudden increase in total backpressure greater than 3000 psi.

SUMMARY

The method outlined in this Application Note quantifies mg/L or lower concentrations of carbachol-fortified eye care products. Separation and detection of carbachol, choline, bethanechol and 2-HPTA using an IonPac CS17 column with 5 mM MSA and suppressed conductivity detection are also examined. Using the method described here, these cholinergic agents are well resolved from commonly occurring inorganic cations. The method demonstrates high precision, high recovery and excellent day-to-day reproducibility for analysis of carbachol.

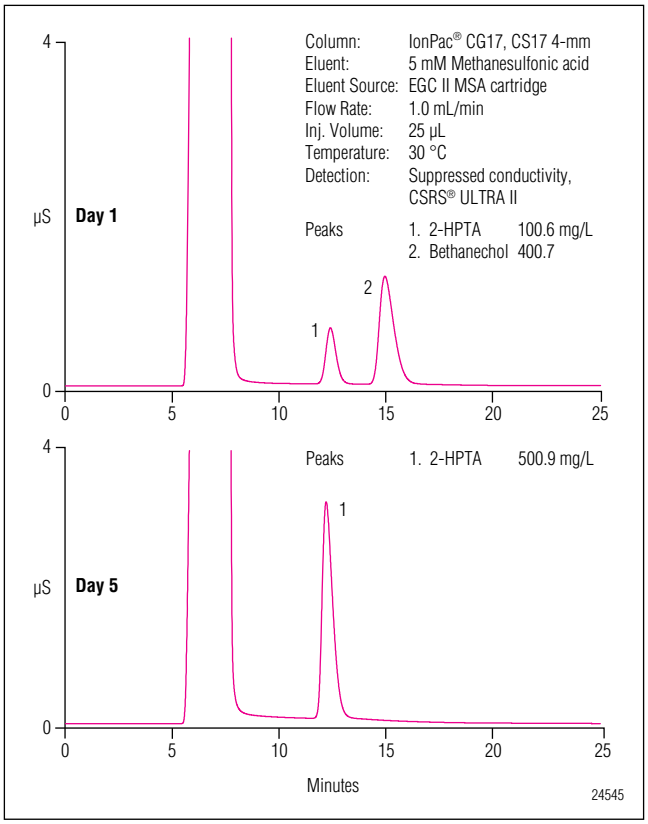

Figure 5. Conversion of bethanechol to 2-HPTA in the presence of 0.1 N NaOH.

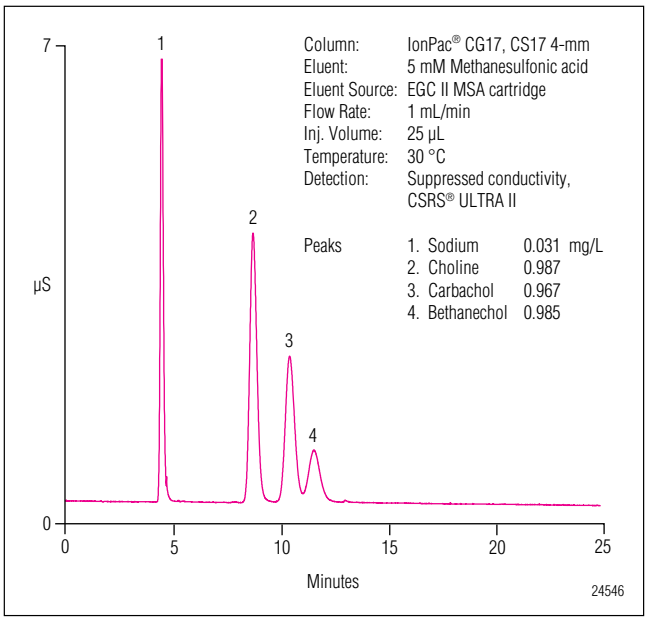

Figure 6. Separation of choline from carbachol and bethanechol in lens solution.

SUPPLIER

U.S. Pharmacopeia, 12701 Twin Brook Parkway, Rockville, MD 20852 USA, (800) 277-8772, www.usp.org

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4. Application Note 124, Determination of Choline in Dry Milk and Infant Formula, Dionex Corporation. LPN 1054.
5. Application Note 148, Determination of Bethanechol by Ion Chromatography Dionex Corporation. LPN 1510.

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