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Application Note 237

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Analysis of Benzalkonium Chloride on the Acclaim Surfactant Column by High-Performance Liquid Chromatography

INTRODUCTION

Benzalkonium chloride, a quaternary ammonium salt with antiseptic properties, is often used as an antiseptic, similar to other cationic surfactants. Its general formula is $[C_6H_5CH_2N(CH_3)_2R]Cl$, where R represents n-C_8H_{17} to n-$C_{18}H_{37}$ (Figure 1). The mode of action of quaternary ammonium compounds appears to be associated with their effect on the cytoplasmic membrane, which controls cell permeability. Extensive data are reported for effective concentrations (0.01%–0.1%) and affected bacterial species. The homologs n-C_{12} and n-C_{14} in benzalkonium chloride products comprise a major portion of the alkyl group.[1] In general, the homolog n-C_{12} is most effective against yeast and fungi, and the homolog n-C_{14} against gram-positive bacteria.[2] Benzalkonium chloride is not effective against gram-negative bacteria, and is not recommended for treatment when sanitization is critical.[3]

Although high-performance liquid chromatography (HPLC) has been used to separate and quantify benzalkonium chloride homologs,[2,4-7] the analysis can be challenging because the analyte peaks often tail on silica-based reversed-phase (RP) columns. This is due to the presence of residual silanols on the silica surface and the high degree of hydrophobicity of the surfactant. In this application note (AN), a complete HPLC methodology for determining benzalkonium chloride homologs in real samples, such as a sterile elastic strip and eye drops, is presented. The HPLC analysis was performed on a specialty column, the Acclaim® Surfactant column, with UV detection. The stationary phase features a unique surface chemistry that effectively deactivates the ionic interaction between the silica surface and cationic surfactants, resulting in excellent peak shapes for cationic surfactants.[8] In this AN, method development, linearity, and the limit of detection are also discussed. The total amount of benzalkonium chloride in sterile elastic strips and eye drops was determined and the amounts of homologs n-C_{12} and n-C_{14} were estimated by area normalization.

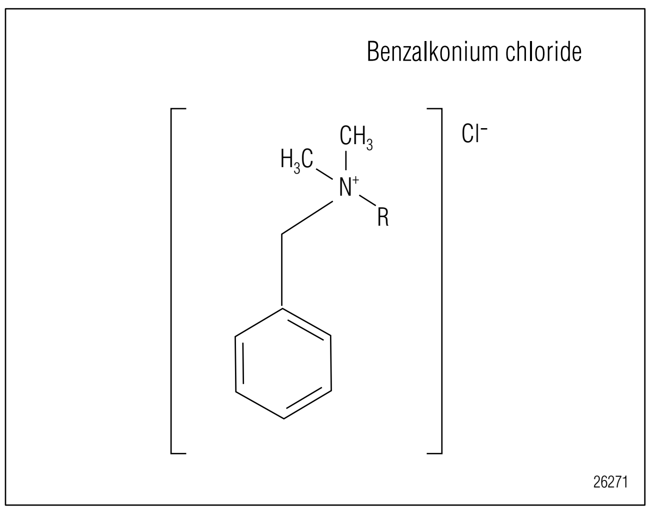

Figure 1. Structure of benzalkonium chloride.

EQUIPMENT

UltiMate® 3000 HPLC system

- DGP-3600 pump with SRD-3600 Solvent Rack with degasser
- TCC-3200 thermostatted column compartment
- WPS-3000TSL autosampler
- VWD-3400 UV-vis detector

Chromeleon® 6.80 SP3 Chromatography Data System

Kudos SK3200LH ultrasonic generator, Kudos Ultrasonic Instrumental Co., Shanghai, China

REAGENTS AND STANDARDS

Deionized water, from Milli-Q® Gradient A10

Acetonitrile (CH_3CN), HPLC grade, Fisher Scientific

Benzalkonium chloride (50% in water, CAS 63449-41-2, PN 09621), Fluka

Formic acid, ≥ 98%, Fluka

SAMPLES

Two sterile elastic strip samples (benzalkonium chloride patches, samples 1 and 2) and two samples of eye drops (samples 3 and 4) were purchased at a local store. Sterile elastic strip samples 1 and 2 were the same brand and product name but purchased from different stores. The two eye drop samples were over-the-counter drugs to which benzalkonium chloride was added as a supplementary component. Sample 3 is sold for anti-eyestrain and sample 4 is sold for allergic conjunctivitis treatment.

CONDITIONS

Chromatographic Conditions

Column:	Acclaim Surfactant column, 5 µm, 4.6 × 250 mm (P/N: 063203)
Column Temp.:	30 °C
Mobile Phase:	A: 100 mM Formic acid (dissolve 1.90 mL formic acid in 500 mL water) B: Water C: Acetonitrile : H_2O (70 : 30, v/v) In gradient (Table 1)
Flow Rate:	1.0 mL/min
Inj. Volume:	5 µL
Detection:	Absorbance at 208 nm (other wavelengths, for example, 225 and 230 nm, may also be used to achieve a better baseline but analyte response is lower)

Table 1. Gradient Elution Program

Time (min)	Formic acid 100 mM (%)	CH_3CN / H_2O (70 / 30, v/v) (%)	H_2O
-5	15	5	80
0	15	5	80
10	15	55	30
15	15	55	30

PREPARATION OF STANDARDS AND SAMPLES

Stock Standard Solution

Measure 2 mL of benzalkonium chloride standard (50% in water) and dilute in a 10 mL volumetric flask with water. The concentration of stock standard solution is 10%.

Working Standard Solutions

Prepare five working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with water. The concentrations of benzalkonium chloride are 20, 50, 200, 500, and 1000 µg/mL, respectively.

Sample Preparation

Add two patches of sterile elastic strips and 20 mL water to a 50 mL conical flask with stopper, and then use ultrasonic extraction for 15 min. Prior to injection, filter the solution through a 0.22 µm filter (Millex-HV).

The eye drop samples can be injected directly without dilution.

Spiked Brand of Sterile Elastic Strip Samples

Add 200 µL of stock standard solution of benzalkonium chloride, two patches of sterile elastic strips, and 19.8 mL water to a 50 mL conical flask with stopper, and then use ultrasonic extraction for 15 min. Filter the solution through a 0.22 µm filter (Millex®-HV) prior to injection.

RESULTS AND DISCUSSION

Retention Behavior of Benzalkonium Chloride on the Acclaim Surfactant Column

The Acclaim Surfactant column features reversed-phase, anion-exchange, and hydrogen-bonding retention mechanisms.[8] These features allow the column to be used differently than a conventional reversed-phase column. Thus, the retention behavior of benzalkonium chloride on the Acclaim Surfactant column was evaluated by varying the selectivity modifiers, ionic strength, pH value, and the amount and type of organic solvent in the mobile phase. Experiments show that increasing the buffer concentration (ionic strength) and buffer pH value produces an increase in retention time; and increasing the proportion of the organic solvent in mobile phase decreases the retention time. The results are consistent with the reported retention behavior of cationic surfactants on the Acclaim Surfactant column.[9]

Method Reproducibility

In the benzalkonium chloride standard (CAS 63449-41-2, Sigma-Aldrich), homolog n-C_{12} is predominant, with homologs n-C_{14} and n-C_{16} also being present.[10,11] Figure 2 shows an overlay of chromatograms of seven benzalkonium chloride standards with concentrations ranging from 20 to 1000 µg/mL. The peak area proportion of homolog n-C_{16} on 1000 µg/mL level (chromatogram 7) is 0.71%, which was calculated by comparing homolog n-C_{16} area to the total peak area of the three homologs; therefore, the amount of homolog n-C_{16} in the standard was ignored in subsequent calculations.

The method reproducibility was estimated by making seven consecutive injections of a 500 µg/mL benzalkonium chloride standard. The retention time reproducibility for both homologs, n-C_{12} and n-C_{14} was 0.107%; the peak area reproducibility for homolog n-C_{12} and n-C_{14} were 0.656% and 1.809% respectively.

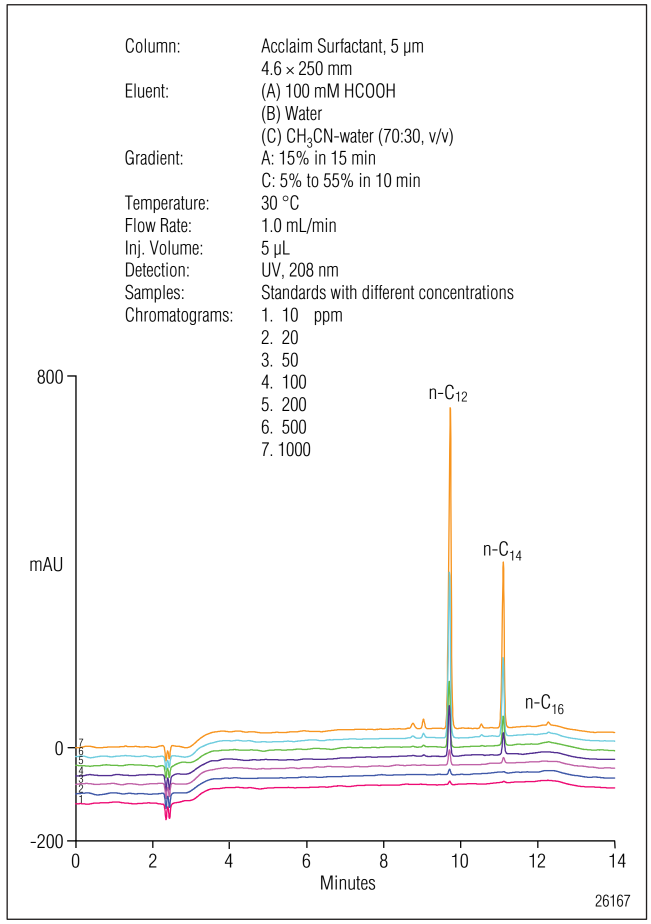

Figure 2. Overlay of chromatograms of seven benzalkonium chloride standards with different concentrations.

Linearity and Method Detection Limit of Benzalkonium Chloride

Calibration linearity for benzalkonium chloride was investigated by making five replicate injections of each of the five standard concentrations. The external standard method was used to establish the calibration curve and to quantify benzalkonium chloride in samples. As shown in Figure 3, excellent linearity was observed from 20 to 1000 µg/mL on plotting the concentration versus the sum of peak areas for homologs n-C_{12} and n-C_{14}. Separate calibration curves for homologs n-C_{12} and n-C_{14} were also prepared. These data are summarized in Table 2.

The detection limits for each of the homologs n-C_{12} and n-C_{14} were calculated using the equation:

$$\text{Detection limit} = S\,t_{(n-1,\,1-\alpha=0.99)}$$

where S = standard deviation of replicate analyses

n = number of replicates

$t_{(n-1,\,1-\alpha=0.99)}$ = Student's value for the 99% confidence level with n – 1 degrees of freedom

Using eight consecutive injections of 500 µg/mL benzalkonium chloride standard to determine the S value, the estimated method detection limits (MDL) were 15 and 12 µg/mL for n-C_{12} and n-C_{14} respectively.

Table 2. Calibration Data

Analyte	Equation	r
Benzalkonium Chloride	A = 0.0763c–1.2514	0.9993
Homolog n-C_{12}	A = 0.0734c–0.7593	0.9980
Homolog n-C_{14}	A = 0.0817c–0.6133	0.9978

Determination of Total Benzalkonium Chloride in Samples

Two sterile elastic strip (samples 1 and 2) and two eye drop (samples 3 and 4) samples were analyzed. The results are summarized in Table 3. Figure 4(A) shows overlays of chromatograms of sample 1 and the same sample spiked with standard. The determined amount is in agreement with the labeled value. Sample 1 was found to contain homolog n-C_{12} but no homolog n-C_{14}. Sample 2, despite being the same brand and same product type as sample 1, may be a faulty product or at least not a very effective one, as no homolog of benzalkonium chloride was found (Figure 4B). Figure 5 shows chromatograms of the eye drop samples. Homologs n-C_{12} and n-C_{14} were found in sample 3, and only homolog n-C_{12} was found in sample 4.

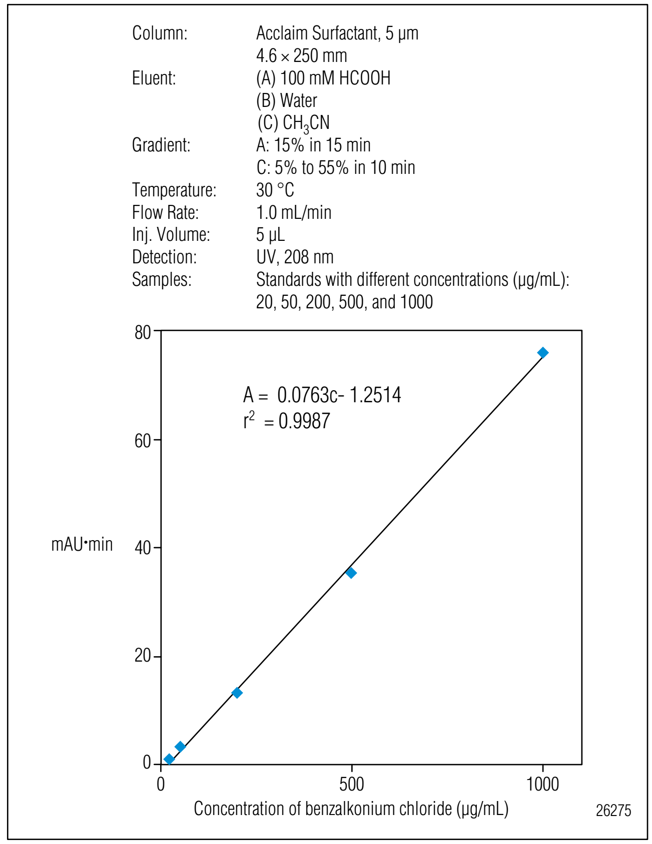

Figure 3. Calibration curve for benzalkonium chloride.

Determination of Homologs n-C_{12} and n-C_{14} of Benzalkonium Chloride in Samples

Because homologs n-C_{12} and n-C_{14} only differ by two units of CH_2, their sensitivity by UV detection may be assumed to be similar. Thus, the proportions of homologs n-C_{12} and n-C_{14} in samples can be estimated using the following formula:[12]

$$Cc_{12} = \frac{Ac_{12}}{Ac_{12} \times Ac_{14}} \times C \quad (1)$$

$$Cc_{14} = \frac{Ac_{14}}{Ac_{12} \times Ac_{14}} \times C \quad (2)$$

where, Ac_{12} and Ac_{14} represent peak areas of homologs n-C_{12} and n-C_{14} respectively; Cc_{12} and Cc_{14} represent concentrations of homologs n-C_{12} and n-C_{14} respectively; and C is the concentration of benzalkonium chloride.

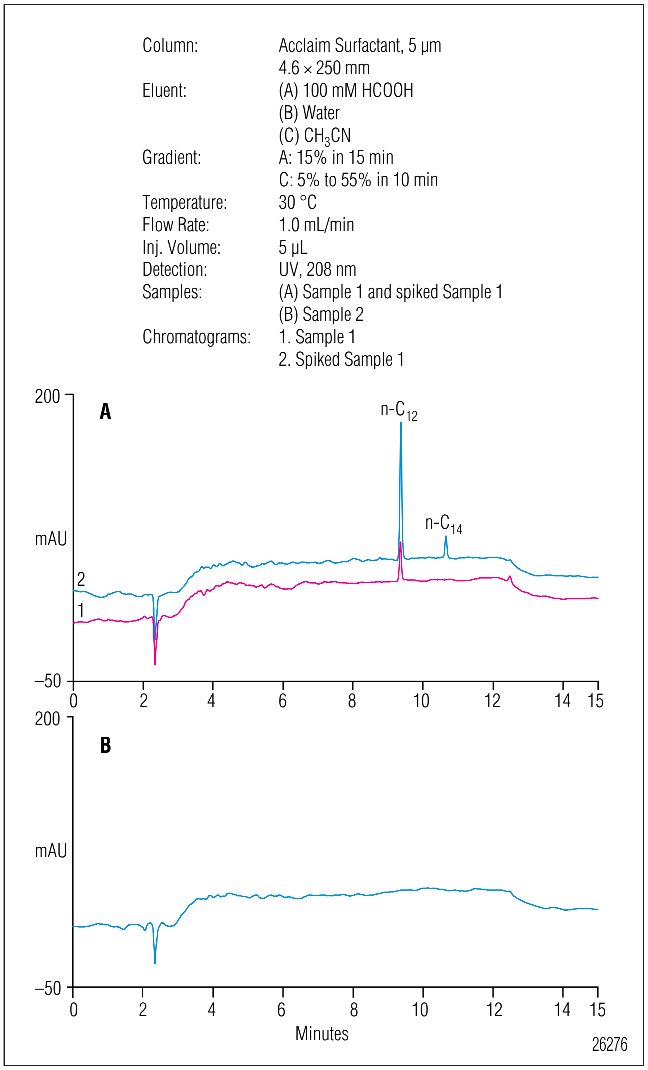

Figure 4. Chromatograms of sterile elastic strips sample (A) 1 and (B) 2.

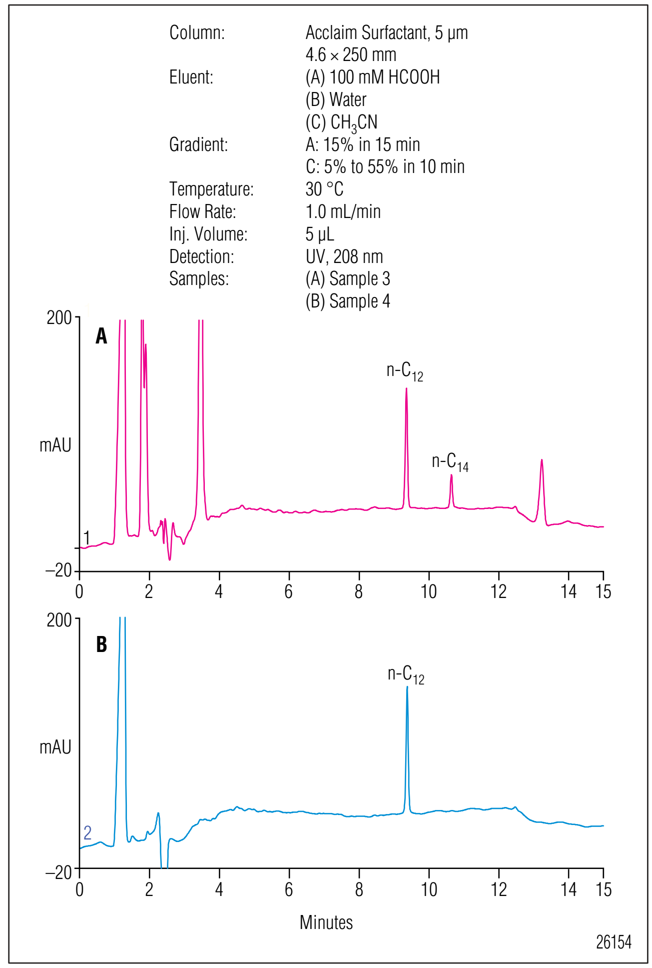

Figure 5. Chromatograms of eye drop sample (A) 3 and (B) 4.

Table 3. Analysis Results for Sterile Elastic Strips and Eye Drops

Analyte	Sterile Elastic Strips						Eye Drops	
	1					2	3	4
	Detected (mg/patch)	Labeled (mg/patch)	Added (µg/mL	Found (µg/mL)	Recovery (%)	Detected (µg/mL)	Detected (µg/mL)	Detected (µg/mL)
Benzalkonium Chloride	0.47	0.5	100	97	97	ND	146	118
Homolog n-C_{12}	0.43		70	83	119	–	116	116
Homolog n-C_{14}	ND		30	25	83	–	32	ND

Table 4. Concentrations of Homologs C_{12} and C_{14} in the Working Standards				
Conc. of Benzalkonium Chloride Standard (µL/mL)	C_{12} Homolog		C_{14} Homolog	
	Proportion	Concentration (µL/mL)	Proportion	Concentration (µL/mL)
20	70%	14	30%	6
50		35		15
200		140		60
500		350		150
1000		700		300

The proportions of homologs n-C_{12} and n-C_{14} in the benzalkonium chloride standard solutions used for calibration were estimated using formulas 1 and 2. The average distribution was 70% for homolog n-C_{12} and 30% for homolog n-C_{14}, based on which concentrations of homologs n-C_{12} and C_{14} in working standards were used to determine calibration linearity (Table 4). The calibration curves for the individual homologs were used to determine the amounts of these homologs in samples 1 through 4 (Table 3).

CONCLUSION

This application note describes an efficient and simple method for the analysis of benzalkonium chloride used as an antiseptic in sterile elastic strips and eye drops. The Acclaim Surfactant column yields efficient and symmetrical peaks for benzalkonium chloride, demonstrating its effectiveness for cationic surfactant analysis.

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