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Indirect Determination of Process Impurity Cetrimonium Bromide in *Neisseria meningitidis* A/C/Y/W-135-DT Conjugate Vaccine by HPAEC-PAD Method

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Abstract

A validated High Performance Anion Exchange- Pulsed Amperometric Detection (HPAEC-PAD) indirect method is described to determine traces of process impurity cetrimonium bromide (CTAB) in *Neisseria meningitidis* A/C/Y/W-135-DT conjugate vaccine. Method reported here, measures the bromide ion present in CTAB. As part of impurity profiling, samples of crude and purified meningococcal four serogroup (A, C, W-135 and Y) polysaccharides and final vialed conjugate vaccine were analyzed for CTAB content. Vaccine grade pure polysaccharides, the control and the final vaccines both formulated in phosphate buffered saline (PBS) showed sub ppm levels of CTAB impurity. PBS used in this work showed detectable bromide levels presumably due to contamination originated from saline. The proposed method is very sensitive (LOD=0.04 ppm, LOQ =0.11 ppm), accurate, reproducible and compatible with polysaccharide and PBS environments.

Keywords: Cetrimide, HPAEC PAD, *Neisseria meningitidis*, Conjugate vaccine, Polysaccharide, Process impurity.

Highlights

- Reported HPAEC PAD method determines CTAB impurity in polysaccharide preparations by measuring bromide ion in it.
- The method is not interfered with PBS and NaCl environment
- Method is highly sensitive with a LOD of 0.04 ppm

Introduction

Hexadecyl (or Cetyl) trimethylammonium (or cetrimonium) bromide (CTAB) is a cationic quaternary ammonium, amphiphillic (with polar head group and hydrophobic carbon tail) surfactant with many applications in biochemistry laboratories, cosmetics and pharmaceutical industry. It is one of the components of the topical antiseptic 'cetrimide' invented and introduced by ICI (Imperial Chemical Industries a former British company) with a brand name Cetavlon. Mainly due to cationic nature of CTAB, it is used to selectively precipitate anionic bio-polymers such as nucleic acids (both plant and animal) and polysaccharides of different microorganisms (example: *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, Typhimurium Vi, Cryptococcus neoformans etc.) [1-5].

Capsular polysaccharides of many encapsulated bacteria are virulence determinants and are good vaccine candidates [2]. Based on these surface antigenic capsular polysaccharide chemical structures and serological properties, for example, 13 serogroups in *Neisseria meningitidis* and 91 serotypes in *Streptococcus pneumoniae* have been identified. Generally in an upstream process of meningococcal or pneumococcal vaccine manufacture, individual serogroup or serotype cultures are grown in separate fermenter batches, and immunogenic high molecular form polysaccharides (PS) are precipitated and isolated from culture supernatants using CTAB [1]. In subsequent purification step a calcium chloride exchange, separates polysaccharide from polysaccharide-CTAB complexes. Later ethanol (≥80%) precipitation step yields relatively pure polysaccharides. Further down-stream polysaccharide purification procedures such as enzyme hydrolyses, ultracentrifugation and multiple diafiltrations remove cellular impurities such as nucleic acids, proteins, endotoxin, and process impurities such as CTAB, formaldehyde [6] etc., to yield ultimately vaccine grade polysaccharides. In a different application of a general flu vaccine preparation [7], influenza virus is purified by chromatography and ultra/diafiltration steps, inactivated by beta propiolactone and disrupted by CTAB (or other suitable detergents) to solubilize the viral surface antigens HA and NA. Polystyrene based resin is used to remove CTAB. However to measure left over trace quantities of CTAB in a process sample, polysaccharide preparation or in a final formulated vaccine a sensitive, robust and product compatible analytical method is required. We developed and report here a validated high performance anion exchange-pulsed amperometric detection (HPAEC-PAD) method to use as a process impurity limit test based on Dionex Application Note 173[8].

Materials and Methods

Materials

Sodium hydroxide 50% w/w ($_{cat\# ss254}$) and HPLC grade water were purchased from Fisher Scientific Inc. (Pittsburgh, PA, USA). CTAB ($_{cat\#\ 57\text{-}09\text{-}0}$), Sodium Bromide ($_{cat\#S4547}$), sodium chloride ($_{cat\#\ 7647\text{-}14\text{-}5}$) were purchased from Sigma Aldrich (St. Louis, MO, USA) and 1x PBS from Corning Cellgro (Manassas, VA, USA).

Preparation of CTAB standard solution

40 mg of CTAB (cat# 57-09-0) was weighed into Fisher 50 ml tube and dissolved in 40 ml of HPLC grade water to prepare a 1000 ppm stock solution of CTAB. Stock solution is stored at 30°C to avoid any precipitation. From the 1000 ppm stock, further diluted to arrive at 100, 10, and 2 ppm working solutions. Standard curve CTAB concentrations of 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0, and 5.0 ppm were prepared from working solutions.

Sodium bromide (Sigma) was used as reference standard to confirm bromide ion retention time. 40 mg of sodium bromide was weighed into a Fisher 50 ml tube and dissolved in 40 ml of HPLC grade water to prepare a 1000 ppm stock solution of sodium bromide. From which was further diluted to arrive at 0.5 ppm and 1 ppm working stocks.

Sodium chloride was used as an interference standard. To prepare 1000 ppm stock solution of sodium chloride 40 mg of sodium chloride was weighed into a Fisher 50 ml tube and dissolved in 40 ml of HPLC grade water and was then diluted to arrive at 0.5 ppm and 1 ppm working stocks.

Chromatographic-conditions

HPAEC-PAD runs were performed on Thermo Scientific ICS 5000 equipped with AS-AP Auto sampler, IonPac AS15 2 x 250 mm Analytical column attached through IonPac AG15 2 x 50 mm Guard column. Electrodes: reference- pH-Ag/AgCl, working-disposable Ag, detector- PAD on cyanide waveform [8]. Eluent A: Water and Eluent B: 200 mM sodium hydroxide. Eluent to have 31.5% Eluent B i.e 63 mM sodium hydroxide. Eluents were blanketed under 4-5 psi nitrogen. Column temperature: 30˚C. Auto sampler tray temperature kept at 25˚C. Flow rate was 0.25 ml/min. 10 µl duplicate injections were performed. Eluents were prepared in degassed Milli-Q water with resistance ≥18.2MΩ.

Crude and vaccine grade polysaccharides

A, C, W135 and Y serogroup polysaccharides of *Neisseria meningitidis* used in this study were produced at JNI Medical Corporation using the modified CTAB precipitation method [1]. Final vialed A/C/Y/W-135-DT conjugate vaccines were formulated to contain 4 µg of each serogroup polysaccharide conjugated to a total of 16-32 µg diphtheria toxoid (DT) in 1xPBS [9]. Polysaccharide samples of crude (80% ethanol pellet, 10 mg/ml PS) and purified vaccine grade polysaccharides (1mg/ml PS), vialed vaccine Lot#JN-NM-001 and one vial of control vaccine Menactra Lot#U4658AA were processed (see section 2.5) and used for measuring CTAB content in them.

Process sample preparation

A 500 µL of process sample (ex. crude or pure vaccine grade polysaccharide or final vaccine vial sample where content of CTAB needs to be estimated) is placed on 1.5 ml 10K spin filter and centrifuged on a bench top mini-centrifuge at 14,500 rpm for 10 minutes at room temperature (22±2˚C) to remove high molecular weight polysaccharide, carrier protein or PS conjugated to DT as retentates. Spiked process samples with known amounts of CTAB were also treated same as above. Test samples then were transferred to HPAEC PAD maximum recovery sample tubes in the auto-sampler and performed 10 µl duplicate injections.

Method validation

In order to validate the proposed HPAEC PAD method, accuracy, precision, specificity, detection limit, quantitation limit, linearity and solution stability parameters were considered as per ICH guidance for industry Q2 (R1) [10-11]. Linearity of the proposed method was tested across the range of standard curve CTAB concentrations of 0.1, 0.2, 0.3, 0.5, 0.75, 1.0, 2.0, 3.0, and 5.0 ppm. The calibration runs were performed by two different technicians on three different days to verify the day to day and person to person variation. Average peak areas vs concentration were plotted, relative standard deviation on responses and correlation coefficient (R^2) were calculated using Microsoft Excel. Detection limit and quantitation limit were calculated from standard curve values and residual standard deviation values from each standard curve points by the formulas LOD=3.3σ/s and LOQ=10σ/s where σ is residual standard deviation and s is the slope of the standard curve. Spike recovery on final vaccine flow through samples were measured by giving 0.5 and 1ppm CTAB spike. Robustness of the method was verified by deliberately introducing variation i) in column temperature 30˚C ±1˚C and ii) in Eluent B NaOH concentration 63±1mM with 0.5 and 1ppm CTAB concentration injections. Sample solution stability was tested for an 8 hour period by repeating duplicate injections before and after leaving the range of samples (0.1, 0.2, 0.3, 0.5, 0.75, 1, 2, 3 and 5ppm) in the auto-sampler compartment at 25˚C for 8h.

Results

Accuracy and specificity of the method was verified with reference standard sodium bromide. Bromide ion present in both NaBr and CTAB eluted at retention time of about 13 min (Figure 1). We have optimized the autosampler tray temperature to keep at 25˚C in order to avoid any precipitation of CTAB in the sample solution. Advantage of the IonPac AS15 column used in this method is chloride (RT~3 min) and bromide (RT~13 min) ions separated with very distinct difference in retention times (data not presented). We observed a clear linearity (R^2>0.99, N=9) over the aqueous CTAB concentration range 0.1 to 5 ppm (Table 1, Figure 2) and average peak areas were used for plotting calibration curve. All the calibration curve points were verified in duplicate injections over three different days and mean peak areas against each concentration were very close (Table 1). When crude or purified polysaccharide aqueous solutions of serogroup A, C, W-135 and Y were used to measure the CTAB impurity in them, the levels of CTAB impurity varied from serogroup to serogroup (Table 2). Crude PSs of serogroups A and C contained comparatively lower CTAB content than W-135 and Y. Purified vaccine grade PSs. (Figure 3) showed sub-ppm CTAB or below detection levels (in serogroup A PS).

When robustness of the method was verified with 0.5 and 1ppm CTAB concentrations by deliberately introducing variation of ±1˚C to optimal column temperature of 30˚C, temperature

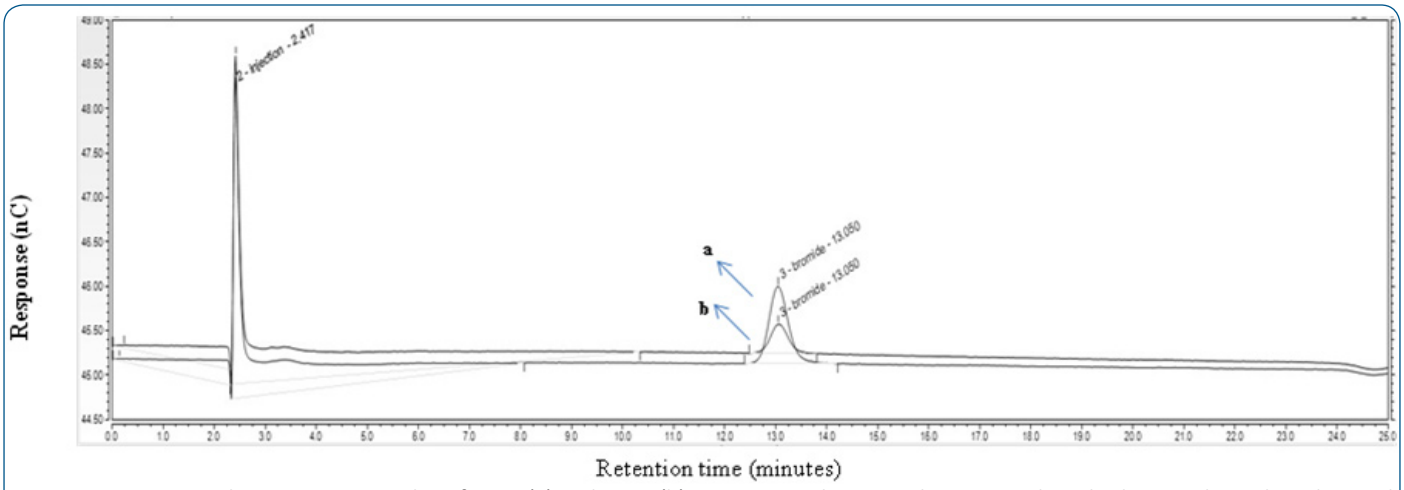

Figure 1: HPAEC PAD chromatogram overlay of NaBr (a) and CTAB (b) at 1 ppm each run on the proposed method. Bromide peak is observed ~13 minutes

Table 1: Increasing concentrations of CTAB 0.1 to 5 ppm range were used for duplicate injections on proposed HPAEC method and obtained mean areas plotted against CTAB concentration to generate the standard curve

Sample	CTAB (ppm)	Mean Area (nC*min) day1	Mean Area (nC*min) day 2	Mean Area (nC*min) day 3
1	0.0	0.0	0.0	0.0
2	0.10	0.029	0.025	0.025
3	0.20	0.057	0.065	0.046
4	0.30	0.073	0.068	0.086
5	0.50	0.126	0.152	0.111
6	0.75	0.187	0.160	0.200
7	1.0	0.250	0.220	0.286
8	2.0	0.440	0.463	0.455
9	3.0	0.740	0.652	0.827
10	5.0	1.187	1.284	1.314

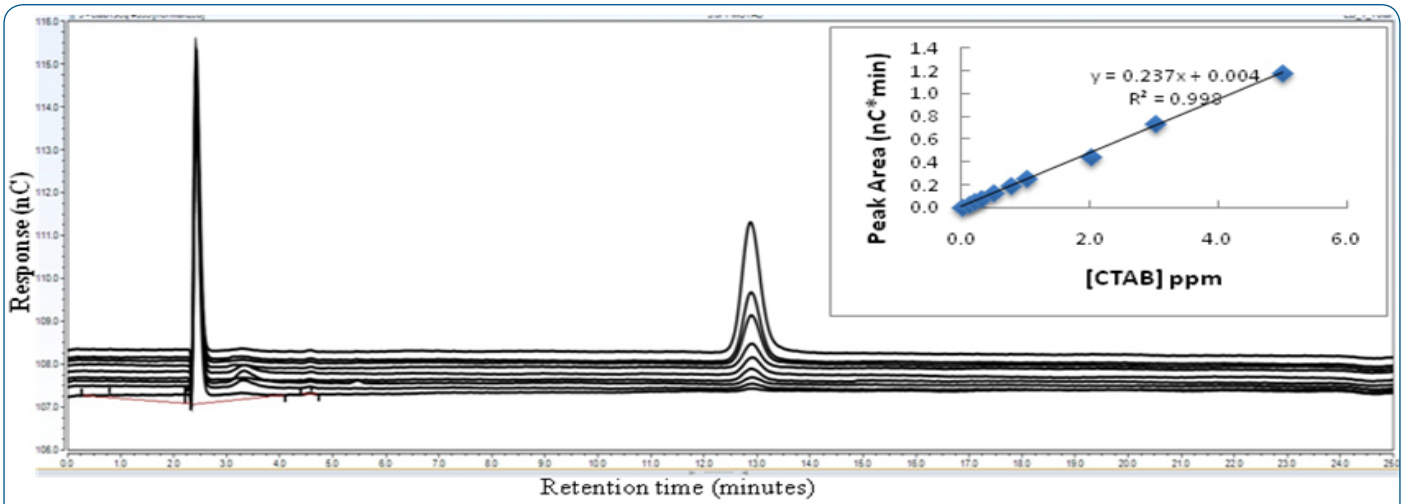

Figure 2: HPAEC PAD chromatogram peak overlay of CTAB concentrations 0.1, 0.2, 0.3, 0.5, 0.75, 1.0, 2.0, 3.0, and 5.0 ppm run on the proposed method and the standard curve (inset) shows CTAB amounts against average peak areas plotted, a clear linearity (R^2>0.99, N=9) was observed

change caused only little deviation in respective retention times and peak areas (Table 3). However when Eluent B concentration changed ±1mM from 63 mM, although retention time of the bromide peak did not get effected much (~1% varied - data not presented) a big (37-41 %) difference noticed in the peak areas on both 0.5 and 1 ppm concentrations of CTAB. This result indicated that Eluent B NaOH concentration should be kept constant to have more reliable and dependable results by this method.

1x PBS control showed an average amount of 1.78 ppm bromide in it. The CTAB (bromide) content in JNNM001 vaccine flow through was derived by subtracting the control PBS bromide and is calculated as 0.69 ppm. Control vaccine Menactra showed

Table 2: CTAB impurity levels in four serogroup polysaccharides crude and pure samples estimated by HPAEC PAD method.

Sample stage	Serogroup	Mean Peak area (nC*min) ±S.D.	CTAB content (ppm)
Crude (80% ethanol pellet)	A	1.51±0.03	6.34
	C	0.56±0.01	2.36
	W-135	2.69±0.11	11.31
	Y	2.22±0.12	9.33
Pure (vaccine grade)	A	0.004±0.001	b.d
	C	0.044±0.01	0.16
	W-135	0.095±0.02	0.38
	Y	0.053±0.01	0.21

b.d- below detection

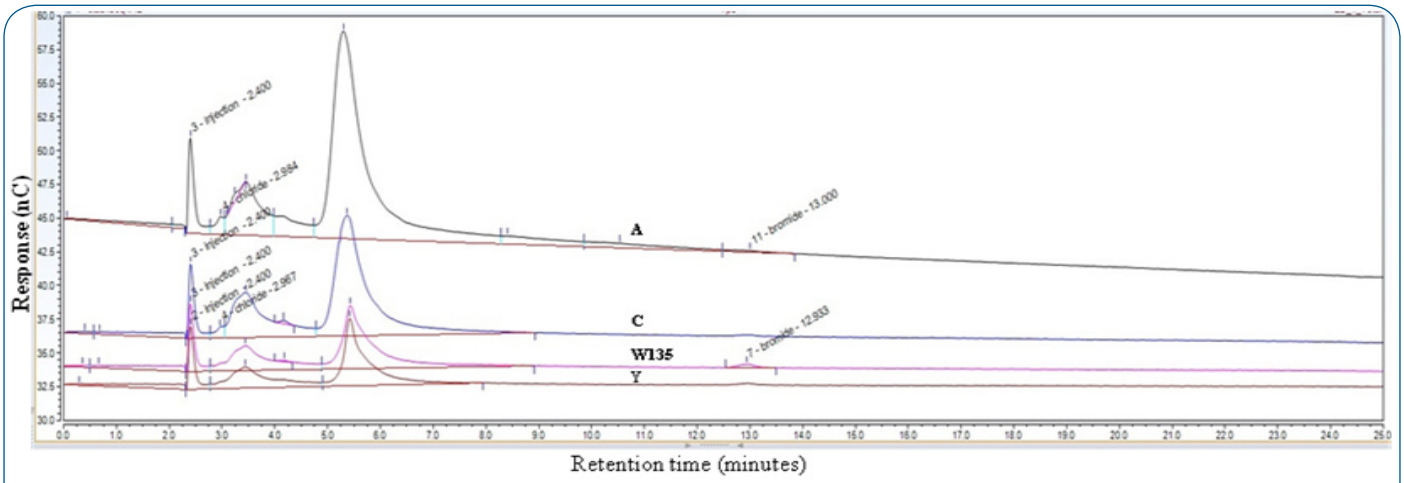

Figure3: HPAEC PAD chromatogram overlay of A, C, W-135 and Y serogroup aqueous solutions of pure polysaccharides (at 1mg/ml) run on the proposed method. Serogroup A polysaccharide showed below detectable level whereas W-135 polysaccharide preparation showed highest level (0.38 ppm) of CTAB (Table 2)

Table 3: To test the robustness of the method a deliberate change ± 1°C in column temperature from optimum 30°C revealed slight variation in the mean retention time and peak areas on duplicate injections at 0.5 and 1ppm CTAB concentrations

Column temperature	29°C		30°C		31°C	
CTAB ppm	0.5	1.0	0.5	1.0	0.5	1.0
Mean Retention time(min)	12.917	12.925	12.934	12.925	12.717	12.717
Mean Peak area (nC*min)	0.137	0.287	0.152	0.286	0.139	0.272

an average amount of 0.95 ppm bromide. Solution stability for 8h was calculated by leaving the samples at 25˚C in the auto sampler compartment. Among the range of concentrations used for solution stability, a range of 5.7 to 16% difference (mean difference 12%) was noticed in the peak area.

When 0.5 and 1 ppm CTAB spiked in the final vaccine flow through samples, spike recovery of 99% and 102% respectively were observed indicating PBS matrix has least interference, although PBS itself exhibited some back ground bromide content may be due to bromide present in saline as a trace contaminant.

Discussion

A recent review covered the occurrence, fate and toxicity of quaternary ammonium compounds in the environment, giving detailed analytical tools to determine such compounds [12]. Currently there is no regulatory guidance available on permissive amounts of CTAB in human vaccines. Different methods at different pharmaceutical contexts were reported in literature to determine CTAB [13-15]. Light absorption behavior of CTAB was reported earlier [16] and reverse phase HPLC methods were published earlier, for the determination of both non-ionic and ionic surfactants in bioprocess intermediates [17]. In another report of reverse phase HPLC method, CTAB and lidocaine were determined simultaneously at 208nm [18]. Polysaccharides show strong absorption at 206 nm, hence to determine accurately the trace amounts of CTAB in a polysaccharide environment, UV absorption methods are not applicable. Determining CTAB (detection limit 2 ppm) by indirect method of bromide ion

estimation by ion chromatography using IonPac AS4A-SC analytical column with conductivity detector was reported earlier [15]. However in their IonPac AS4A-SC method separation between chloride and bromide ions was poor. In the current HPAEC PAD method we used cyanide waveform that is optimized for cyanide, but it can also detect sulfide, chloride, bromide and thiosulfate with a convenient separation on IonPac AS 15 analytical column [8]. We could get increased sensitivity (detection limit 0.04 ppm) by PAD detection. Also AS15 column improved separation (about 10 min apart) between chloride and bromide making this method more compatible with chloride common contaminants in the sample matrix.

Conclusion

The validated HPAEC PAD indirect method described in this report measured process impurity CTAB in crude and vaccine grade A, C, W135 and Y serogroup polysaccharides of *Neisseria meningitidis* and also in final vialed conjugate vaccine samples used in this study. The proposed method works by measuring bromide ion in CTAB, in the cyanide waveform. The method is sensitive and compatible with polysaccharide and chloride in the sample matrix.

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