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Application of capillary ion electrophoresis and ion chromatography for the determination of O-acetate groups in bacterial polysaccharides

Robert W. Hepler, Charlotte C. Yu Ip*

Department of Virus and Cell Biology, Merck Research Laboratories, West Point, PA 19486, USA

Abstract

Many bacterial polysaccharides possess O-linked acetate groups as constituents of their repeating units which often can serve as immunological determinants. It is therefore important to develop analytical methods for process monitoring as well as product characterization when such O-acetylated polysaccharides are used as components of vaccines. This is the case in a polysaccharide conjugate vaccine under development for treatment of diseases caused by *Streptococcus pneumoniae*. An ion chromatographic (IC) method utilizing suppressed conductivity detection (SCD) was developed to quantitatively measure O-acetate groups in the capsular polysaccharides from *S. pneumoniae* types 18C and 9V following hydrolytic release of O-acetate from the polysaccharide backbones using 2 m*M* sodium hydroxide. IC was carried out using an OmniPac PAX-500 column and 0.98 m*M* NaOH in 2% methanol as the mobile phase. Capillary ion electrophoresis (CIE) with indirect photometric detection was evaluated as an alternative method. The CIE method utilized a 72 cm × 75 μm I.D. fused-silica capillary and an electrolyte composed of 5 m*M* potassium hydrogenphthalate, 0.5 m*M* tetradecyltrimethylammonium bromide, and 2 m*M* sodium tetraborate, pH 5.88. A comparison of CIE and IC–SCD in terms of reproducibility, accuracy, linearity, and sensitivity will be presented.

1. Introduction

Purified type-specific capsular polysaccharides from *Streptococcus pneumoniae* are currently used in the preparation of a commercially available vaccine against pneumococcal (Pn) disease. The Pn polysaccharides are also conjugated to carrier proteins as a Pn conjugate vaccine for the prevention of pediatric Pn diseases [1]. Until recently, routine characterization of the carbohydrate composition of the vaccine was accomplished by colorimetric assay. Currently, the technique of high-pH anion-exchange chromatography (HPAEC) using pulsed amperometric detection (PAD) is used for the analysis of the carbohydrate composition of acid-hydrolyzed Pn polysaccharide types [2]. HPAEC–PAD allows for the detection of subnanomole amounts of the monosaccharide components of these Pn polysaccharides. However, the constituent side chain

* Corresponding author.

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groups such as O-acetate in Pn polysaccharide 9V and Pn polysaccharide 18C cannot be detected by HPAEC–PAD. The objective of this work was to investigate analytical methods for the separation and quantitation of O-linked acetate in Pn polysaccharides and to determine the optimal hydrolysis conditions for the release of O-acetate from Pn polysaccharide types 9V and 18C.

Determination of the O-acetyl content of bacterial polysaccharides has traditionally been accomplished by chemical assay [3] or, more recently, by NMR techniques [4]. The application of ion chromatography (IC) coupled with suppressed conductivity detection (SCD) for the quantitative analysis of O-linked acetate in Pn polysaccharides has not been reported, although the technique of IC–SCD has been used to quantitate inorganic and organic anions with sensitivity in the parts-per-billion range [5]. We initially investigated the use of IC–SCD as a quantitative tool to monitor hydrolytic release of O-acetate from Pn polysaccharides.

Recent developments in the use of capillary ion electrophoresis (CIE) using indirect photometric detection [6–9] provided an alternative method for the detection and quantitation of O-acetate groups in hydrolyzed Pn polysaccharide types 9V and 18C. This method involves the incorporation of a cationic surfactant in the running buffer to cause a reversal of the normal electroosmotic flow [9,10]. The method also involves the reversal of the polarity of the CE system to cause the migration of anions from the site of injection (cathode) past the detector window to the anode. Detection is accomplished by incorporation of a highly-UV absorbing species in the buffer. Analyte ions displace chromophoric buffer ions as they pass the detector, causing a reduction in absorbance which can be recorded as a positive peak by reversing the polarity of the detector output.

In this article, we will investigate the applicability of the recently developed CIE methodologies for the analysis of O-acetate in bacterial polysaccharides and compare this new method with the IC method.

2. Experimental

2.1. Ion chromatographic systems and eluents

Ion chromatography (IC) was performed on a Dionex (Sunnyvale, CA, USA) BioLC equipped with a gradient pump module and connected to a pulsed electrochemical detector operating in the conductivity mode. Separation was accomplished using Omnipac-PAX 500 guard and analytical columns (250 mm × 4 mm I.D., 8.5 μm bead diameter) and an anion trap column (ATC-1) which was placed between the gradient pump and injection valve to remove anionic contaminants from the eluent. Chemical suppression of background conductivity was accomplished using a Dionex Anion Micro Membrane Suppressor (AMMS-II) operated via an AutoRegen pump with 50 m*M* sulfuric acid as the regenerant. Samples were injected via a Spectra Physics SP8880 autosampler equipped with a PEEK stator and rotor seal (Rheodyne) and a 100-μl PEEK sample loop. Data were collected by a PE Nelson A/D converter (Cupertino, CA, USA) and analyzed using PE Nelson Turbochrom software version 3.3.

IC was performed by isocratic elution with 0.98 m*M* sodium hydroxide in 2% methanol for 15 min at a flow-rate of 1 ml/min. This was followed by a 10-min wash in 50 m*M* NaOH, 2% methanol, and a 12.5-min re-equilibration with the starting buffer. The conductivity detector was set to a range of 3 μS. The background conductivity for the isocratic buffer was less than 1 μS at a regenerant flow-rate of approximately 10 ml/min.

2.2. Capillary ion electrophoresis system

Capillary ion electrophoresis (CIE) was performed on an Applied Biosystems (Foster City, CA, USA) Model 270A CE unit equipped with an 72 cm × 75 μm I.D. (50 cm separation length) fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) and an UV detector. The electrophoresis buffer was composed of 5 m*M* potassium hydrogenphthalate, 4 m*M* sodium

tetraborate, and 0.5 m*M* tetradecyltrimethylammonium bromide (TTAB) pH 5.88. The buffer was filtered through a 0.45-μm filter prior to use. Each run consisted of a 3-min wash with 0.1 *M* sodium hydroxide, a 3-min wash with high purity water, and an equilibration with running buffer for 5 min prior to sample injection. Samples were loaded using a 5-s vacuum injection at 127 mm of mercury. The detector was set at 254 nm with the output polarity reversed. The detector range was set at 0.004 OD units. The samples were separated over a period of 10 min at an applied voltage of −15 kV.

2.3. Reagents and preparation of standards and samples

Moisture corrected samples of type-specific Pn polysaccharides were prepared from purified Pn polysaccharide powders obtained from the Merck Manufacturing Division (West Point, PA, USA). An EM Sciences Aquastar V3000 volumetric Karl Fisher titrator was used for moisture content analysis. Moisture corrected samples of polysaccharide powder (2.5 mg/ml) were dissolved in pyrogen-free water over a period of 24 h at room temperature on a rotating platform.

Sodium hydroxide, sodium acetate trihydrate, and HPLC-grade methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sodium tetraborate, TTAB, potassium hydrogenphthalate, and N-acetylmannosamine were purchased from the Sigma (St. Louis, MO, USA).

An acetate standard was prepared by dissolving sodium acetate in water at a concentration of 10 m*M*. This standard was stored at −20°C in 1-ml aliquots; 100 μl of this concentrated standard was diluted in a total volume of 2 ml of water for a concentration of 500 μM. Five serial 2-fold dilutions of this standard were performed to prepare the remaining concentrations of the standard curves.

2.4. Alkaline hydrolysis of bacterial polysaccharides

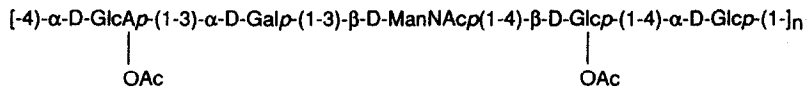
For the assay validation studies, 100 μl of a 2.5-mg/ml solution of moisture corrected Pn polysaccharide type 18C was mixed with 250 μl of pyrogen-free water and 50 μl of freshly prepared and helium sparged 16 m*M* sodium hydroxide for a final concentration of 0.625 mg/ml polysaccharide and 2 m*M* NaOH. The samples were incubated overnight at room temperature and diluted in water 1:1.3, 1:5, and 1:15 prior to analysis.

For the quantitation of O-acetate in Pn 9V and 18C, 50 μl of a 2.5-mg/ml solution of polysaccharide was mixed with 125 μl of pyrogen-free water and 25 μl of 16 m*M* sodium hydroxide for a polysaccharide concentration of 0.625 mg/ml and a hydroxide concentration of 2 m*M*. The samples were incubated at room temperature for 16 h, diluted 1:10, and 1:5 (type 9V, and 18C, respectively) in water, and analyzed by CIE and IC using the methods outlined above.

3. Results and discussion

The repeating units of Pn polysaccharides types 9V and 18C are shown in Fig. 1 and are based on structures published by previous investigators [11–14]. Repeat unit molecular masses of 968 and 1006 for Pn polysaccharides 9V and 18C, respectively, were used for conversion of mass to moles. While Pn polysaccharide 18C was found to contain one O-acetate group per polysaccharide repeating unit [11], the reported number of O-acetate groups per repeating unit in Pn polysaccharide 9V has varied from 1.2 using NMR [13] to 1.6 using a colorimetric assay [12], suggesting either inaccuracy in determination, or more likely the existence of a mixed population of singly and doubly-acetylated molecules. For this report, alternative methods for the quantitation of O-acetate in Pn polysaccharides 9V and 18C which require little sample and are relatively easy to perform were investigated for the characterization of these components of a Pn conjugate vaccine. Both methods involve the release of O-acetate from the polysaccharide backbone followed by chromatographic or electrophoretic

Pn Ps Type 9V



Pn Ps Type 18V

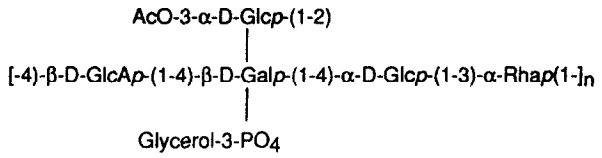


Fig. 1. Structures of the repeating units of the capsular polysaccharides (Ps) of *Streptococcus pneumoniae* types 9V and 18C.

separation and detection. A typical example of an electropherogram and chromatogram using the methods outlined in the experimental section are shown in Figs. 2 and 3. Fig. 2 is a CIE electropherogram of a sample of hydrolyzed Pn polysaccharide 18C in which the acetate migrates as a sharp peak with a migration time of 7.2 min, the broad (9.0 min) peak following acetate is caused by the presence of carbonate in the sample. Fig. 3 is the IC chromatogram of the same hydrolyzed Pn polysaccharide 18C sample in which acetate elutes as a sharp peak with a retention time of 5.8 min.

3.1. Optimization of the hydrolysis conditions for the release of O-acetate from Pn polysaccharidess 9V and 18C

Preliminary experiments were performed using IC to determine the hydrolysis conditions necessary for the removal of O-acetate from these polysaccharides. Various concentrations (1 m*M* to 50 m*M*) of sodium hydroxide were used for different lengths of time (0.5 h to 48 h) at different temperatures (4°C to 65°C). It was determined that 2 m*M* sodium hydroxide at room temperature for 16 h provided optimum

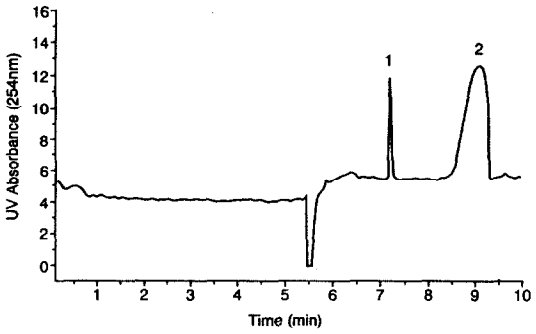


Fig. 2. Typical electropherogram of an alkaline hydrolyzed Pn polysaccharide 18C showing O-acetate released from the sample. Peaks: 1 = acetate; 2 = carbonate.

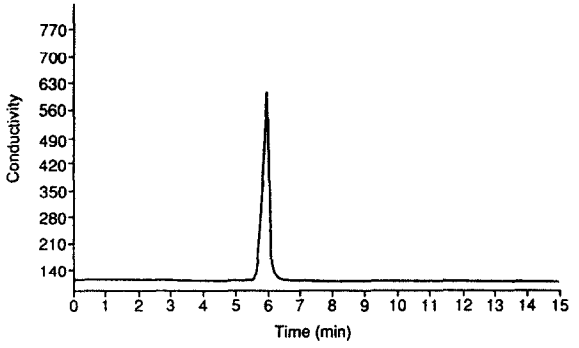


Fig. 3. Typical chromatogram of an alkaline hydrolyzed Pn polysaccharide 18C showing O-acetate released from the sample.

release of O-acetate from Pn polysaccharide 9V and Pn polysaccharide 18C. A time-course study was also performed using CIE in which samples were incubated in 2 m*M* NaOH for 0.5 h to 48 h at room temperature. The results of this study are shown in Fig. 4. Again, 16 h at room temperature in 2m*M* sodium hydroxide were sufficient to provide maximum release of O-acetate from Pn polysaccharide 9V and Pn polysaccharide 18C.

In order to verify that the hydrolysis conditions chosen for O-acetate removal did not release N-linked acetate (Pn polysaccharide 9V contains an N-acetate group in addition to the O-acetate groups, see Fig. 1), we also subjected N-acetylmannosamine (N-acetylated monosaccharide) and Pn polysaccharide type 4 (which contain both N-acetylmannosamine and N-acetylfucosamine as part of the constituents in the repeating unit [15]) to the hydrolysis conditions outlined above. No acetate was detected in either the Pn polysaccharide 4 or N-acetylmannosamine samples after 48 h of hydrolysis in 2 m*M* NaOH (see Fig. 4).

One disadvantage of the IC method for O-acetate quantitation was the inability to quantitate background levels of acetate (free residual acetates from purification process) in samples.

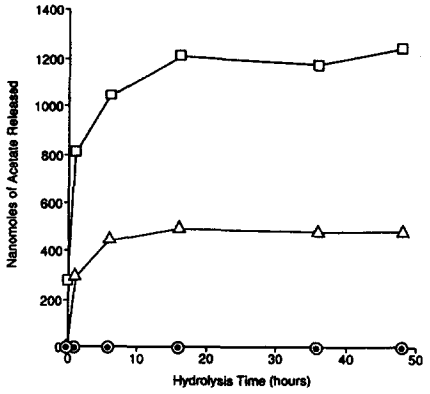


Fig. 4. Time course of O-acetate released from Pn polysaccharide 9V, □; Pn polysaccharide 18C, ○; N-acetylmannosamine, △; and Pn polysaccharide type 4, ●.

This was due to the fact that the IC buffer contained 0.98 m*M* sodium hydroxide, which resulted in on-column hydrolysis of O-acetate in the samples during the chromatographic procedure. This was not a problem with CIE because the CIE buffer did not contain sodium hydroxide and therefore did not hydrolyze the samples during analysis. For these reasons, the IC method is limited to the analysis of final purified samples or samples which have been dialyzed free of acetate prior to hydrolysis and IC.

3.2. Linearity

Table 1 gives the linearity data as obtained using CIE and IC. Standard curves were generated using a sodium acetate standard at concentrations of 500, 250, 125, 62.5, 31.25, and 15.6 μM in both the CIE and IC assay. Peak area by CIE appears linear (on a linear scale) throughout the range of acetate concentrations used in this study, and preliminary data showed peak areas to be linear through acetate concentrations as high as 5 m*M*. Peak area by IC is linear (on a linear scale) up to a concentration of 250 μM acetate.

Fig. 5 shows the relationship between acetate concentration and migration time for CIE. The data show that migration time decreased with increasing concentrations of acetate. This is due to electromigrative dispersion of the sample [7]. We therefore chose to limit the standard curve in a typical assay to 500 μM in order to minimize excessive broadening of the migration zones.

Table 1
Linearity data for CIE and IC

Method	r^a	Linear range (μM)
CIE	0.9999	15.6–500
IC	0.9988	15.6–250

[a]Linear correlation coefficient.

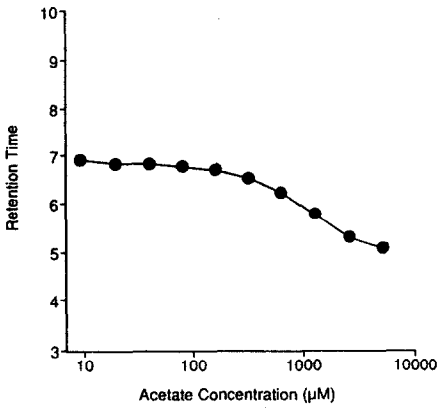


Fig. 5. Effect of acetate concentration on capillary ion electrophoretic retention time.

3.3. Limit of detection and sensitivity

Limits of detection for both assays were calculated based on a method [16] wherein the limit of detection, y, is equal to the blank (equivalent to the y intercept or y_B), plus three times the standard deviation of the blank, S_B. The limits of detection for CIE and IC determined by this method were 7 μM and 12 μM, respectively. It should be noted at this point that although the limits of detection for both methods appear similar, the absolute amount of acetate detected is much smaller using capillary electrophoresis. Assuming that a volume of 3.5 nl/s (value provided by the manufacturer) is taken up by the capillary, the mass amount of acetate applied to the capillary at the lowest standard concentration is $1.1 \cdot 10^{-3}$ nmol. The mass of acetate applied to the IC column at the lowest standard concentration is $1.56 \cdot 10^{-1}$ nmol so that overall CIE is two orders of magnitude more sensitive than IC on a mass basis.

3.4. Repeatability and reproducibility

In order to arrive at estimates of assay repeatability (intra-day precision) and reproducibility (inter-day precision), a hydrolyzed sample of Pn polysaccharide 18C was assayed at three dilution levels; corresponding to the upper (480 μg/ml), mid (125 μg/ml), and lower (42 μg/ml) regions of the standard curve using five sequential injections on three days. The relative standard deviations (R.S.D.) for intra- and inter-day variability were calculated and are presented in Table 2.

The 3-day averages of the within-run variability for CIE at the 1:1.3, 1:5, and 1:15 dilutions were 4.6, 4.5, and 4.3%, respectively. The 3-day average within-run values for IC were 0.7, 0.6, and 0.9% for the 1:1.3, 1:5, and 1:15 dilutions, respectively. As shown in Table 2, the results indicate that the total variability for these two assays are similar.

The reproducibility of the migration/retention time for each of the methods was also determined from the data outlined above. The average R.S.D. for the migration time of acetate by CIE ($n = 15$) on each of three days was 1.2%, while the overall variability in migration time across three days of analyses was 1.6%. The corresponding data for IC retention time was 0.6% and 1.0% for the 3-day average R.S.D. and overall variability, respectively.

Table 2
Assay variability (% R.S.D.) data for CIE and IC

Dilution level	Day 1		Day 2		Day 3		Total (inter-day)	
	CIE	IC	CIE	IC	CIE	IC	CIE	IC
1:1.3	6.5	0.3	2.3	1.0	5.1	0.7	7.2	3.4
1:5	5.1	0.5	5.3	0.5	3.0	0.8	6.2	1.1
1:15	3.7	1.1	5.5	0.8	3.7	0.9	2.6	5.0

3.5. Assay comparison

In order to compare the two assays, a sample of sodium hydroxide-hydrolyzed Pn polysaccharide 18C was diluted to achieve an equivalent acetate concentration of approximately 250 n*M*/ml. This solution then was used to prepare dilutions equal to 90, 80, 70, 60, 50, 40, 30, 20, and 10% of the stock. Each sample was then assayed in duplicate by CIE and IC along with acetate standards. Interpolated concentrations for each sample then were calculated based on the acetate standards normalized to the point of each curve, and compared by plotting the values obtained from IC on the x axis and the values from CIE on the y axis. The slope of the line is 0.997 ± 0.023, and the y intercept is 0.92, indicating that each method yields equivalent results over this concentration range.

3.6. Matrix effects

In order to identify potential effects of the sample matrix on the outcome of routine analyses, a spiking experiment was performed on each of three days. In this experiment, a hydrolyzed Pn polysaccharide 18C sample or water was spiked with five levels of acetate, 20, 40 80 160 and 320 μM. The slope of the lines representing the acetate standard in water and the acetate standard spiked into the sample matrix were calculated and shown in Table 3. The data showed that the slopes were the same (within-assay variability) for the total acetate content in either matrix using CIE or IC. This result indicates that the recovery of acetate in water is the same as in a sample matrix containing hydrolyzed Pn polysaccharide 18C and that there are no detectable matrix effects in either assay under these conditions.

Table 3
Matrix effect data for CIE and IC

Matrix	Slope	
	CIE	IC
H_2O	232 ± 1	20407 ± 527
Hydrolyzed sample	237 ± 5	20959 ± 481

3.7. Quantitation of O-acetate in Pn 9V and 18C

In order to determine the molar ratio of O-acetate to polysaccharide in the Pn polysaccharides 9V and 18C, triplicate hydrolyses were performed and analyzed in duplicate by both CIE and IC. The μM of acetate determined in these assays were divided by the μM amount of each of the polysaccharide to provide a ratio of O-acetate to polysaccharide repeating unit. The CIE data shows that Pn polysaccharide 9V contains 1.4 mol of O-acetate per mole of polysaccharide repeating unit. Pn polysaccharide 18C was found to contain 0.8 mol of O-acetate per mole of polysaccharide repeating unit. These values are in general agreement with structural studies of Pn polysaccharides 9V and 18C as reported by previous investigators [12–13]. IC provides essentially the same information; however, because IC cannot be used to determine free acetate in the samples prior to hydrolysis, the values obtained by IC in cases where samples contain free acetate would overestimate the actual O-acetate content. Free acetate values can be determined by CIE and were taken into account in the calculations used to provide the data presented above.

4. Conclusions

Alternative methods for rapid determination of the O-acetate content of two type specific polysaccharides from *S. pneumoniae* using both IC and CIE following alkaline hydrolysis have been described and compared. Both IC and CIE demonstrate similar levels of performance in terms of sensitivity, repeatability, reproducibility and accuracy. However, to avoid the over-estimation of acetate for those Pn polysaccharide samples containing traces of free acetate, the IC method requires that the samples be dialyzed prior to the alkaline hydrolysis procedure. This is due to on-column hydrolysis of O-acetate from

the Pn polysaccharides in the NaOH containing IC eluent. The CIE method is linear to 5 m*M* acetate while the IC method is linear to 250 μM. In addition, CIE has several advantages over IC, which include the elimination of the expensive IC columns which require extensive cleaning and regeneration resulting the generation of large volumes of hazardous waste. Overall run times for each method are 21 and 37.5 min for CIE and IC, respectively. Therefore, we conclude that the CIE method is faster, easier to use and cost effective, and that CIE should be the method of choice for the quantitation of O-acetate in bacterial polysaccharides following alkaline hydrolysis.

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