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Quantification of each Serogroup Polysaccharide of *Neisseria meningitidis* in A/C/Y/W-135-DT Conjugate Vaccine by High-Performance Anion-Exchange Chromatography-Pulsed Amperometric Detection Analysis

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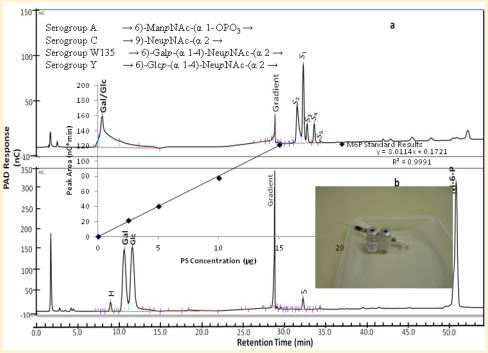
ABSTRACT: Invasive bacterial meningitis caused by *Neisseria meningitidis* can be prevented by active immunization with meningococcal polysaccharide or polysaccharide−protein conjugate vaccines. In a tetravalent A/C/Y/W-135-DT meningococcal conjugate vaccine vial, or in a final formulated bulk, accurate identification and quantification of each polysaccharide are critical in product release. Determination of sialic acid serogroups (C, W-135, and Y) unambiguously is complex since all these serogroups contribute to the sialic acid monosaccharide peaks that overlap in the high-performance anion-exchange chromatography−pulsed amperometric detection (HPAEC-PAD). We report a quantification method that involves generation of monosaccharide standard plots for respective sugars mannosamine-6-phosphate, sialic acid, galactose- and glucose-derived from hydrolysis of mixtures of the four serogroups A, C, W, and Y reference polysaccharides. These plots were then used to obtain the unknown polysaccharide concentrations of A/C/Y/W-135 in vialed vaccine or from formulated final bulks. We also present our results of the HPAEC-PAD profiles on groups C, W-135, and Y polysaccharides when hydrolyzed individually and/or in mixtures to discuss the individual sialic acid peak contributions.



Neisseria meningitidis is a Gram-negative diplococcus that causes life-threatening invasive disease such as meningitis and sepsis. Out of 13 serogroups identified globally, 5 serogroups (A, B, C, W-135, and Y) cause almost all invasive meningococcal infections. Currently licensed meningococcal tetravalent vaccines protect against four serogroups A, C, W135, and Y. These four clinically important meningococcal serogroups express structurally different capsular polysaccharides (shown below) that determine their distinct serological properties.

serogroup A → 6) − Man*p*NAc − (α1 − OPO_3→

serogroup C → 9) − Neu*p*NAc − (α2→

serogroup W135 → 6) − Gal*p* − (α1 − 4) − Neu*p*NAc − (α2→

serogroup Y → 6) − Glc*p* − (α1 − 4) − Neu*p*NAc − (α2→

A meningococcal polysaccharide vaccine has been available for many years that can offer protection against groups A, C, Y, and W135.[1] Recently, improved conjugate vaccines came into existence in which meningococcal polysaccharides are covalently coupled to a carrier protein such as diphtheria toxoid.[2−6] Polysaccharide (PS) amounts in such conjugate vaccines are generally 10-fold less (4 to 5 μg each serogroup PS/500 μL dose) than quantities present in polysaccharide vaccines (approximately 50 μg of each serogroup PS/500 μL dose). Accurate quantification of each polysaccharide present in such low quantities in the final bulk formulations of multivalent conjugate vaccine or in the vial is critical for batch release and poses a challenging task for analytical chemists of vaccine manufacturing companies. Current typical quantification analysis of polysaccharides in vaccine formulations involves two major steps: (i) a hydrolysis step to release monosaccharides followed by (ii) resolving and quantifying monosaccharides (by CarboPac PA1 column or equivalent) with a high-performance anion-exchange chromatographic (HPAEC) method, using pulsed amperometric detection (PAD) to enable high sensitivity (can detect picomole quantities of sugars) and repeatability.[7,8] In such methods, identification of componential polysaccharides is simultaneously achieved by establishing the identity of monosaccharide PAD signatures by correlating with standard sugar peaks. Hence, these methods can replace the traditional time-consuming enzyme-linked immunosorbent assay (ELISA)-based methods to identify individual polysaccharides. Introduced by Hardy et al. in 1988,[9] a high-performance anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) method started the application for monosaccharide analysis. Ricci et al.[10] used the HPAEC-PAD method for quantification of meningococcal group

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A polysaccharide, reporting with optimized hydrolysis conditions on this group A polysaccharide. Since then, this method rapidly gained importance and is now applied for other meningococcal serogroups and other polysaccharides,[8] thereby circumventing the interference problems involved in existing colorimetric methods.

Meningococcal serogroups C, W, and Y contain *N*-acetylneuraminic acid (or sialic acid) that needs mild hydrolysis conditions (weaker acid/lower temperature) to depolymerize the source polysaccharide to release componential sialic acid without much chemical damage (degradation) in order to quantify by subsequent HPAEC-PAD analysis. Sialic acid containing serogroups C, W-135, and Y are more challenging to quantify since all these three serogroups contribute the sialic acid monosaccharide peak(s) that overlap on the HPAEC chromatogram. A previous patent application publication (US2012/0171241 A1) described a method of quantifying saccharides of serogroups W-135 and Y by measuring separately the molar contents of galactose and glucose (Glc), respectively, and then subtracting the results from the total molar quantity of sialic acid derived from W-135, Y, and C in separate HPAEC-PAD runs.[11] Quantification of W and Y polysaccharide by releasing galactose and glucose in harsh hydrolysis and subsequent HPAEC-PAD analysis was reported earlier.[12]

In the current communication, we report a method to quantify individual serogroup polysaccharides of a formulated ACWY-DT tetravalent conjugate vaccine that does not require the subtraction of W or Y quantities. Instead, in this procedure different amounts of well-characterized reference standard meningococcal polysaccharide mixtures of serogroups A, C, W-135, and Y were hydrolyzed by either a harsh method [4 M trifluoroacetic acid (TFA) at 100 °C for 3 h] to release deacetylated mannosamine 6-phosphate from serogroup A,[10] galactose and glucose, respectively, from serogroups W and Y or a mild method (4 N acetic acid at 80 °C for 3 h) to release sialic acid from serogroups C, W, and Y. Then the derived monosaccharides were resolved on HPAEC-PAD to evaluate their corresponding monosaccharide peak areas. Monosaccharide peak areas versus amount of polysaccharide were plotted to generate standard curves for each monosaccharide, namely, mannosamine-6-phosphate (Man*N*-6PO4), galactose, glucose, and sialic acid. Later, vialed vaccine or final formulated bulk were separately hydrolyzed and analyzed for componential peak areas. By plotting these peak areas on standard plots, unknown vial or bulk concentrations of respective polysaccharides were calculated. We also present our results on HPAEC-PAD profiles of polysaccharides, when mild-hydrolyzed individually and/or in mixtures to understand the individual sialic acid peak contributions.

EXPERIMENTAL SECTION

Sodium hydroxide solution 50% w/w (catalogue no. SS254) and sodium acetate trihydrate (catalogue no. BP334) were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Mannose-6-phosphate (catalogue no. M-6876), galactose, glucose, and sialic acid monosaccharide used as standards and trifluoroacetic acid were purchased from Sigma-Aldrich (St Louis, MO). Amicon Ultra10 kDa molecular weight cut-off (MWCO) membrane spin filters were purchased from Millipore Corporation, Billerica, MA. HPLC-grade water and acetonitrile were purchased from Fisher Scientific.

Vaccine grade serogroup A/C/W-135/Y polysaccharides and respective diphtheria toxoid monoconjugates, formulated bulk, and vialed vaccines were produced at JNI Medical Corporation's vaccine manufacturing and product development facility (Omaha, NE) by JN patented technology.

Serogroup A polysaccharide quantification was performed using phosphate estimation,[13] and C, W, and Y polysaccharide quantification was based on sialic acid estimation by resorcinol method.[14] Protein content in monoconjugates, final bulk (formulated and ready to vial), or vialed vaccine were estimated using BSA standard and bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL) following the supplier's recommendations.

Moisture content in individual serogroup (purified and lyophilized) polysaccharide powder was estimated by Karl Fisher method at Analytical Services, University of Iowa Pharmaceuticals.

Formulated Vaccine. Final bulk or vialed conjugate vaccine were formulated in a 1× phosphate-buffered saline (PBS) based on polysaccharide or protein quantification by colorimetric assay methods mentioned in the materials and methods section on concentrated monoconjugate level and concentrated formulated bulk. A/C/Y/W-135 -DT conjugate vaccine was formulated to have 4 μg (±20% acceptance range) of each polysaccharide conjugated to approximately a total of 16−32 μg of diphtheria toxoid.

Monosaccharide (Sigma) mixtures of galactose, glucose, mannose-6-phosphate, and sialic acid at a concentration 0.08 mg/mL were prepared in HPLC-grade water and were run with an isocratic/gradient 80 min method on HPAEC-PAD (details given below), using waveform "Gold Standard PAD" with description carbohydrates (stad.quad.potential). Mannose-6-phosphate (Sigma) was used as a standard as it has similar retention time on CarboPac PA1 column to that of mannosamine-6-phosphate.[10]

JNI-Standard. A/C/W-13/Y polysaccharides of 1 mg/mL solutions were prepared in HPLC-grade water after considering the moisture content in individual lyophilized polysaccharides.

HPAEC-PAD Runs. were performed on Thermo Scientific ICS5000 equipped with Dionex Carbohydrate Certified disposable gold electrode on electrochemical detector in the pulsed amperometric mode with an Ag/AgCl reference electrode and BioLcCarboPac PA1 (4 × 250 mm) analytical column with a Dionex CarboPac BioLc (4 × 50 mm) guard column. The system was run, and the chromatograms were processed on Chromeleon, version 7.0.

Eluents Used. Eluent A: water; eluent B: 5 mM sodium acetate and 100 mM NaOH; and eluent D: 100 mM NaOH, and 250 mM NaOAc. Eluents were prepared with degassed Milli-Q water with resistance ≥18.2 MΩ.

Elution Method. The total run time was 80 min; 0−20 min eluents A (84%) and B (16%) were run in an isocratic manner. From 20 to 50 min, eluent D was gradually increased to 84% in a continuous gradient manner. D (84%) and A (16%) were maintained from 50 to 65 min, and then turned D back to 0% in the 66th minute, keeping eluents A (84%) and B (16%) continuing further until 80 min. The flow rate was 1 mL/min. Eluents were kept under a N_2 blanket during runs.

Hydrolysis Conditions and Safety Considerations. All the polysaccharide hydrolysis steps were handled under a fume hood to avoid exposure to TFA.

Harsh Hydrolysis Method. To release Man*N*-6PO4 from serogroup A or to release galactose or glucose from W135 or Y, respectively, a harsh hydrolysis method was used. In this method, known amounts of polysaccharide or mixture of polysaccharide

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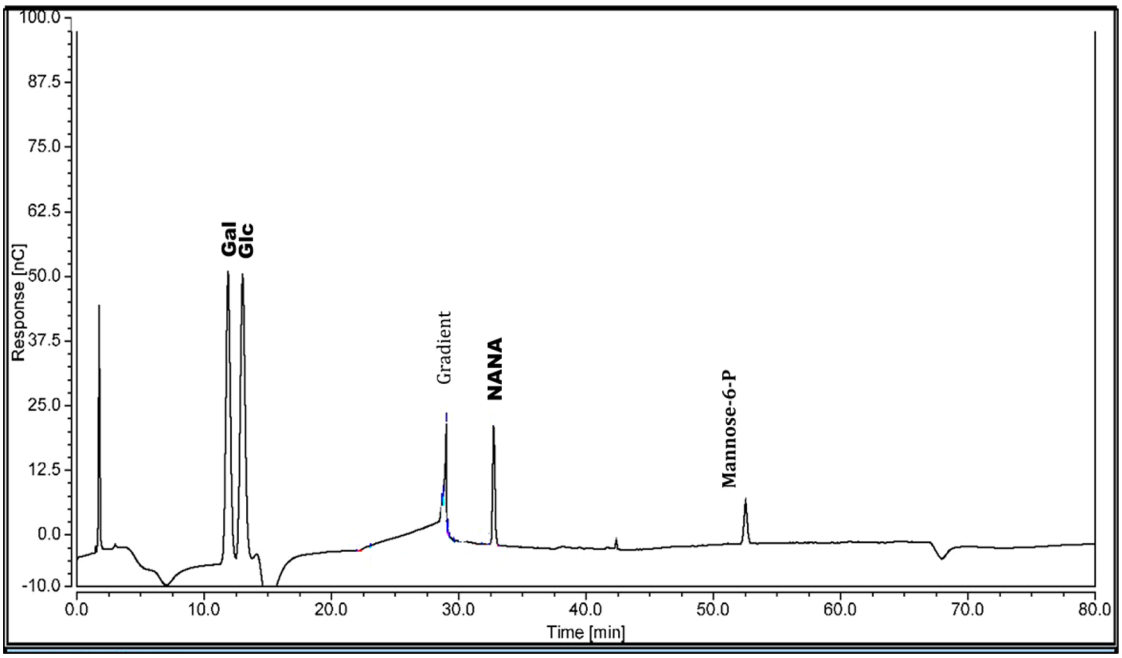

Figure 1. HPAEC-PAD profile of monosaccharide standard (Sigma) mixture run on 80 min isocratic gradient run used in this study. Galactose (11.85 min) and glucose (13.0 min) were seen in the isocratic region; *n*-acetyl neuraminic acid (NANA, 32.72 min) and mannose-6-phosphate were eluted in the gradient phase.

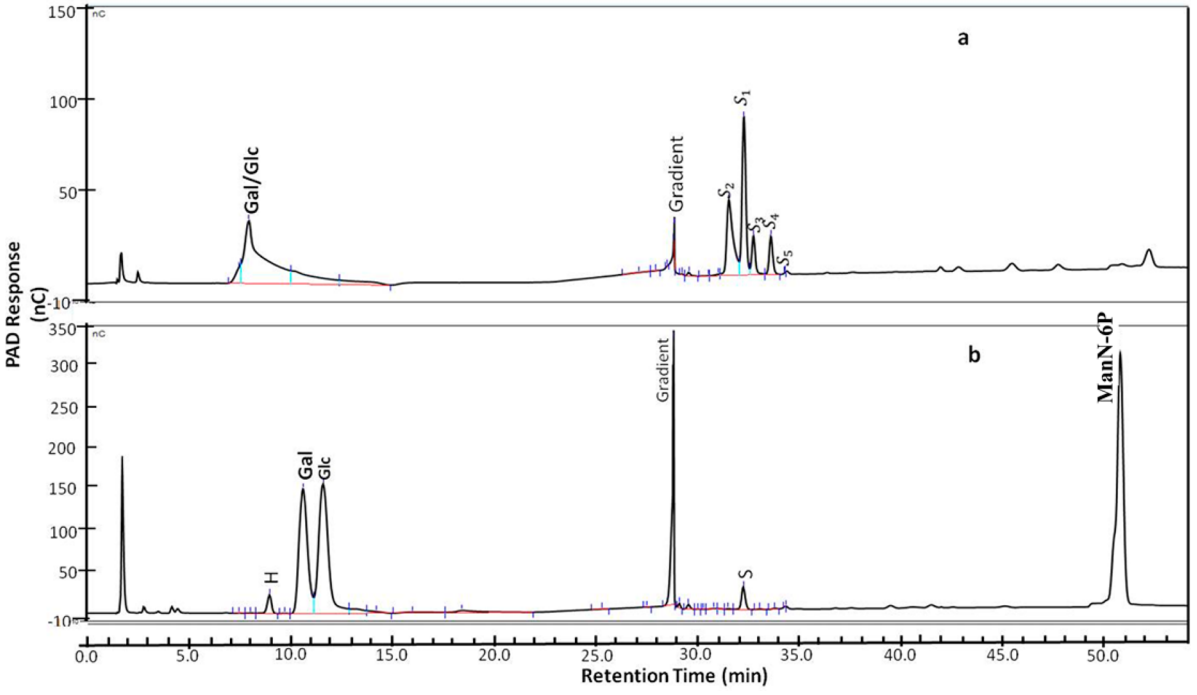

Figure 2. Hydrolyzate HPAEC-PAD chromatograms compared: A/C/W/Y polysaccharide mixtures (20 μg each PS) were hydrolyzed by either (a) mild hydrolysis or (b) harsh hydrolysis. Sialic acid gave multiple PAD responses in mild hydrolysis, whereas in harsh hydrolysis it perished more than 99%.

taken into a Pyrex 13 × 100 mm glass culture tube with phenolic rubber lids. To this, 1:1 v/v of 4 N TFA was added, and the lid was closed and placed on a heating blot slot at 100 °C for 3 h. After the hydrolysis step, the contents in the tube were evaporated using a gentle nitrogen flush on a Thermo ReactiVap system. Later, excess TFA was expelled by adding 200 μL of 50% isopropanol followed by nitrogen flush to dryness. The dried monosaccharide product was solubilized in 250 μL of HPLC-grade water and duplicate injections were injected onto a 50 μL loop using a manual injection port.

Mild Hydrolysis Method. This method is similar to the above method, except the change being 4 N acetic acid and a 80 °C temperature was used instead of a 4 N TFA at 100 °C.

Vial or Final Bulk Hydrolysis. Vaccine from a vial (500 μL) is drawn out with a 1 mL syringe (or directly pipetted 500 μL from formulated bulk) and placed on a 10 kDa membrane spin

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filter to spin at 15 K for 15 min to concentrate to approximately 40 μL. After the spin, the retentate was recovered by a spinning inverted filter in a fresh-collection centrifuge tube. A 40 μL water wash of the filter was also used to collect the residual retentate contents, and the volume was filled up to 100 μL with water, transferred to a 13 × 100 mm hydrolysis tube, and either harsh or mild hydrolysis followed. Polysaccharide repeat unit molecular weights for serogroup A were taken as 341 g/mol[15] and for serogroup C as 338 g/mol.[16] With the strains, we used the polysaccharide *O*-acetyl content (by ^{1}H NMR), consistently estimated low for W-135 (around 27%) and high (around 96%) for serogroup Y. On the basis of this, we calculated the repeat unit molecular weight from the structure of nonacetylated repeat unit ($C_{17}H_{27}NO_{13}$)[17] for W-135 (453 g/mol) and from monoacetylated repeat unit ($C_{19}H_{29}NO_{14}$) for serogroup Y (495 g/mol), and these estimates were used to calculate μ molar quantities present in the final bulk or vial.

RESULTS

Hydrolyzate Chromatograms. In the HPAEC-PAD chromatogram profile (presented in Figure 1) of the standard monosaccharides (Sigma) mixture run on an 80 min long isocratic gradient method, neutral sugars appeared in the isocratic region, galactose at 11.85 min and glucose at 13.0 min, whereas acidic sugar sialic acid (*N*-acetyl neuraminic acid) (NANA) appeared at 32.72 min immediately after the gradient (with eluent D) started and mannoseamine-6-phosphate (Man*N*-6PO4) appeared much later at 52.5 min. There were dips noticed (~15 min retention time) on the baseline of the run and may be due to a possible oxygen dip[18] in the isocratic region of the run, as a result of dissolved oxygen in the sample.

Table 1. Mixtures of A/C/W/Y Polysaccharides of 20 μg each were Hydrolyzed in Two Different Hydrolysis Conditions and Resultant Monosaccharides were Separated on Isocratic Gradient Method and Retention Times and Peak Areas Tabulated

hydrolyzed PS	retention time (min)	area (nC min)
	(a) mild hydrolysis A/C/W/Y	
sialic acid S1, S2, S3, S4, and S5	31.584, 32.334, 32.800, 33.650, 34.434	15.387, 19.110, 4.469, 4.280, 0.389
	(b) harsh hydrolysis A/C/W/Y	
H	8.950	5.5309
galactose	10.600	74.2777
glucose	11.600	89.2448
S	32.300	6.0019
Man*N*-6PO4	50.517	128.4447

Mild hydrolysis on the mixture of A/C/W/Y (each 20 μg) polysaccharide yielded HPAEC-PAD profiles (Figure 2) in which sialic acid exhibited multiple peaks (Figure 2a), whereas harsh hydrolysis condition yielded monosaccharides galactose, glucose and mannosamine-6-phosphate (Man*N*-6PO4) as distinct single peaks (Figure 2b) on our 80 min isocratic gradient method. Respective monosaccharide retention times and peak areas were tabulated in Table 1. Galactose and glucose were eluted in the isocratic region of the run, whereas sialic acid and Man*N*-6PO4 were eluted in the gradient with a sodium acetate push. A gradient transitional peak was observed consistently at approximately 28.6 min in our method as the eluent D (100 mM NaOH and 250 mM sodium acetate) reached a certain percentage in the gradient. Presence of tiny sialic acid peak S (32.367 min) in the harsh hydrolysis profile indicated the extent of perishable nature of sialic acid at harsher hydrolysis conditions. We also noticed an indicator peak H (9.367 min) for the extent of completeness of harsh hydrolysis, which indicates the unhydrolyzed W/Y contents.

Sets of JN-standard A/C/W-135/Y polysaccharide mixtures containing 1.75, 2.5, 5, 10, 15, or 20 μg of each were taken into initial 100 μL starting volumes to subject to two sets of hydrolysis conditions either harsh or mild. Derived monosaccharides were separated on the 80 min long isocratic gradient method in duplicate injections. Each PS representative monosaccharide peak area (mean ± S.D.) values of duplicate injections (on the *Y* axis) versus the amount of PS (on the *X* axis) were plotted to generate four calibration curves. The respective PAD response peak area data points, curve equations, and R^2 were tabulated as Table 2.

Vial or Final Bulk Quantification. Four samples of bulk vaccine and two vials of final vaccine were used for each of the two hydrolysis methods for quantification purposes. Amounts (mean ± S.D) of individual polysaccharides in vial or formulated bulks derived from standard calibration plots using the respective monosaccharide areas (mean ± S.D) were tabulated in Table 3.

Sialic Acid PAD Responses. Polysaccharide hydrolyzate profile derived from C were compared with individual hydrolyzate profiles of either W or Y, presented in Figure 3. Five

Table 2. Sets of JN-Standard A/C/W/Y Polysaccharide Mixtures Containing 1.75, 2.5, 5, 10, 15, or 20 μg of each were Taken into Initial 100 μL Starting Volumes to Subject to Two Sets of Hydrolysis Conditions Either Harsh or Mild[a]

	peak area (nc min) mean ± SD of duplicate injections			
	harsh hydrolysis			mild hydrolysis
concentration of PS (μg)	Man*N*-6PO4	galactose	glucose	sialic acid
0	0	0	0	0
1.75	N/A	14.92 ± 0.15	15.42 ± 0.08	N/A
2.5	18.99 ± 0.34	16.28 ± 1.32	21.15 ± 2.94	31.12 ± 1.34
5.0	37.55 ± 1.87	37.81 ± 2.56	37.28 ± 3.86	53.76 ± 1.77
10	64.79 ± 2.0	N/A	N/A	106.11 ± 3.96
15	94.79 ± 3.48	94.61 ± 1.97	111.41 ± 4.05	155.35 ± 4.74
20	128.92 ± 4.34	131.87 ± 4.57	141.62 ± 3.63	212.11 ± 4.74
curve equation	Y = 6.28x+2.56	Y = 6.41x+2.00	Y = 7.09x+2.23	Y = 10.42x+1.89
R^2	0.9979	0.9971	0.9987	0.9962

[a]PAD response (mean ± S.D.) of duplicate injections for each representative monosaccharide vs amount of PS that were used to generate calibration plots, respective equations, and R^2 of calibration curves tabulated. N/A = not applicable.

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Table 3. Amounts of Individual Polysaccharides (mean ± S.D.) Calculated from Calibration Curves were Tabulated along with Their Respective Monosaccharide Peak Area (mean ± S.D.) Obtained on the Final Formulated Bulk or Vial Componential Hydrolysis and Subsequent HPAEC-PAD Analysis[a]

bulk (n = 4 for each hydrolysis method) vial (n = 2 for each hydrolysis method)	hydrolysis condition (H/M)	representing monosaccharide	area (nC min) mean ± S.D.	concentration of PS determined by standard plot	concentration of PS (μM ± S.D.)
bulk	H	galactose	29.46 ± 1.85	4.29 ± 0.30	0.0095 ± 0.00063
vial	H		29.76 ± 3.74	4.33 ± 0.59	0.0096 ± 0.00122
bulk	H	glucose	25.72 ± 0.78	3.32 ± 0.11	0.0065 ± 0.00056
vial	H		28.12 ± 4.11	3.65 ± 0.58	0.0070 ± 0.00113
bulk	H	Man*N*-6PO4	45.95 ± 1.71	4.21 ± 0.42	0.0123 ± 0.00012
vial	H		47.23 ± 1.31	4.06 ± 0.07	0.0172 ± 0.00049
bulk	M	sialic acid	41.26 ± 2.52	3.36 ± 0.26	0.0099 ± 0.00076
vial	M		49.48 ± 1.65	4.19 ± 0.16	0.0123 ± 0.00049

[a]Molar quantities (μM ± S.D.) were calculated based on respective repeat unit molecular weights.

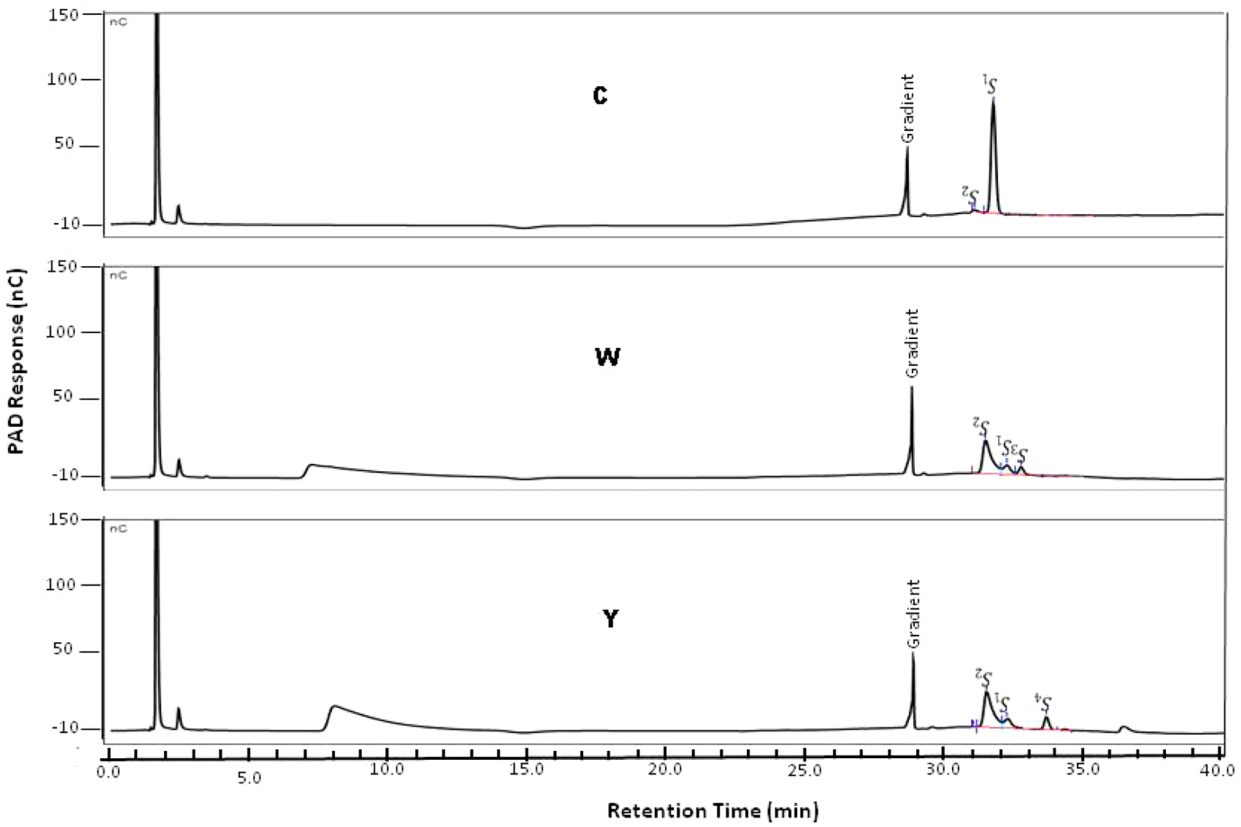

Figure 3. Five micrograms of each polysaccharide C or W or Y individually hydrolyzed in a mild hydrolysis method and examined the hydrolyzate HPAEC-PAD chromatogram profiles for comparison. C alone gave major S_1 and minor S_2 (Figure 4C); W gave S_1 plus two small S_2 and S_3 (Figure 4W); and Y gave S_1 plus two small S_2 and S_3 peaks (Figure 4Y).

microgram serogroup C alone was hydrolyzed, and the hydrolyzates were analyzed (Table 4) on HPAEC-PAD in order to visualize the sialic acid responses generated out of this sialic acid homopolymer. Group C (Figure 3C) upon mild hydrolysis yielded predominantly (S_1 99.45%) a peak at 32.467 min, leaving minor peak (S_2 0.55%) at 31.717 min, indicating the near complete hydrolysis of polysaccharide. Retention time of the S_1 peak matched with standard sialic acid. W or Y individual mild hydrolysis profiles clearly showed that sialic acid species released during mild hydrolysis were different than S_1, indicating incomplete hydrolysis of these disaccharide repeat polymers. When polysaccharide combinations (Figure 4) of C + W, C + Y, W + Y, C + W + Y, and A + C + W + Y were hydrolyzed, there were different PAD responses of sialic acid species at slightly different retention times (labeled as SA $_{1-5}$). Respective peak areas observed were presented in Table 5. The C + W hydrolyzate profile revealed an increase in the SA_1 and SA_2 intensities and the occurrence of SA_3 (at 32.6 min) as a tiny peak; the C + Y hydrolyzate showed the SA_1 and SA_2 increase similar to C + W, but instead of SA_3, there was a new SA_4 observed at 33.567 min. C + W + Y showed cumulative intensity on SA 2, 3, and 4, and a new SA_5 showed up at (33.117 min). In the A + C + W + Y hydrolyzate run, the PAD pattern was identical to the C + W + Y pattern, as there was no additional contribution of sialic acid from serogroup A PS.

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Table 4. Five Micrograms of Each Serogroup Polysaccharides of Groups C, W, and Y Individually Hydrolyzed Using Mild Hydrolysis Method, Derived Monosaccharide HPAEC PAD Peak Retention Times and Peak Areas Tabulated

serogroup (5 μg each)	sialic acid peak	retention time (min)	area (nC min)
C	**S1**	32.467	16.175
	S2	31.717	0.0948
total area			16.270
W	S1	31.950	2.388
	S2	31.150	9.756
	S3	32.517	1.826
total area			13.970
Y	S1	31.867	2.334
	S2	31.117	10.038
	S4	33.367	2.208
total area			14.580

DISCUSSION

To our knowledge, there are no published HPAEC-PAD direct methods available for the quantification of meningococcal polysaccharides in conjugate vaccines other than the patent application referred to above. Talaga et al. (2002) explained the HPAEC-PAD method of pneumococcal polysaccharide quantification in polysaccharide and conjugate vaccines. They observed that creating calibration curves for individual polysaccharide for routine analysis was difficult; for one reason, this could be higher pneumococcal serotype number (about 11) and more complex polysaccharide structures. Since our meningococcal vaccine's tetravalency is reasonably manageable and polysaccharide structures are relatively simpler [either homopolymers (group C or A) or disaccharide repeat units (W or Y)], we chose to prepare individual calibration curves for four-derived monosaccharides from hydrolysis of multiple mixtures of polysaccharide quantities. However, the more delicate situation in handling meningococcal polysaccharides over pneumococcal polysaccharides is the presence of sialic acid, in the former, which is a more labile sugar. In this method of meningococcal polysaccharide quantification, two hydrolysis conditions and one HPAEC-PAD run-method would suffice to quantify the vial material, hence requiring two vials of 500 μL doses one for each hydrolysis condition. Five-hundred microliter vial content or the same volume of final bulk were concentrated by 10 kDa MWCO spin filters to facilitate manageable hydrolysis reaction volumes to avoid usage of large volumes of acid and to avoid excess time required for large volumes to concentrate by nitrogen flush. Phospho-*N*-acetyl mannosamine (phospho-ManNAc) polymeric serogroup A polysaccharide hydrolysis under harsh conditions produced data to build a most dependable standard curve of all the four serogroups, although R^2 values of the other curves also looked acceptable. Since these calibration curves were derived using respective polysaccharide contents on the *X* axis, final vial quantities of each polysaccharide can be achieved without any further subtractions. The HPAEC-PAD, 80 min run on harsh hydrolysis sample exhibited neutral sugars galactose and glucose in the isocratic region of the run where there was no sodium acetate push. Man*N*-6PO4 gave a peak around 52.5 min

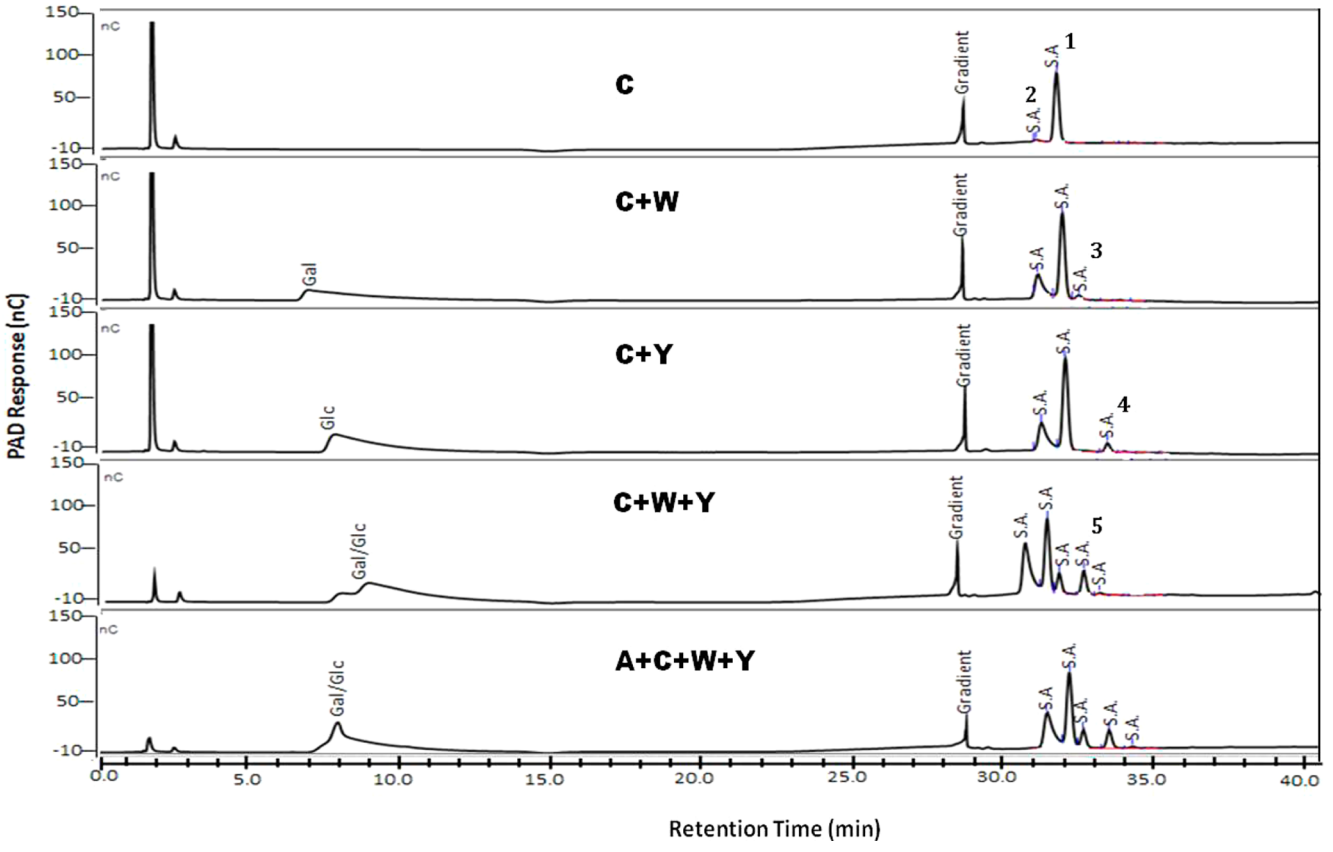

Figure 4. HPAEC-PAD chromatogram overlay of hydrolyzates compared for sialic acid signatures in the following order: C polysaccharide alone, C + W, C + Y, C + W + Y, and A + C + W + Y. Sialic acid signatures released from C alone showed SA 1 and SA 2, where in C + W chromatogram SA 2 increased and 3 appeared new. In C + Y, SA4 appeared new. In C + W + Y, SA 5 appeared new.

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Table 5. Five Micrograms of each Polysaccharide in Combinations of C + W, C + Y, C + W + Y, and A + C + W + Y were Hydrolyzed and Resultant Monosaccharide HPAEC-PAD Peak Retention Time and Peak Areas Tabulated in Comparison with C Alone Values

serogroup (each 5 μg)	sialic acid peak	retention time (min)	peak area (nC min)
C	S1	32.467	16.175
	S2	31.717	0.0948
total area			16.270
C + W	S1	32.050	21.304
	S2	31.234	11.076
	S3	32.600	1.447
total area			33.827
C + Y	S1	32.150	23.772
	S2	31.350	12.443
	S4	33.567	2.198
total area			38.413
C + W + Y	S1	31.384	17.251
	S2	30.634	16.247
	S3	31.800	4.837
	S4	32.617	4.650
		33.117	0.518
total area			43.505
A + C + W + Y	S1	32.334	19.110
	S2	31.584	15.387
	S3	32.800	4.469
	S4	33.650	4.280
	S5	34.434	0.389
total area			43.635

in the gradient region with a 250 mM sodium acetate push. Accuracy of this PS standard curve method of vial quantification mainly depends on the accurate measurement of moisture content in the standard polysaccharide, which in turn determines the accuracy of standard polysaccharide solution used. Four bulk samples and two vial samples were used for each hydrolysis method to confirm the quantification of polysaccharides in the final vaccine. Vaccines were formulated based on colorimetric estimations of individual polysaccharides to contain 4 μg/dose, with an accepted range of ±20%. Average values of (bulk and vial) on each polysaccharide estimated by the current HPAEC-PAD method were comparable to expected formulated values and are within the accepted limits. This method also was tested for intraday reproducibility by repeating both the hydrolysis methods and found very reproducible. Although we presented our work on conjugate formulation, this method can be applied for meningococcal polysaccharide vaccines.

Mild hydrolysis condition of 2 N acetic acid (AcOH) and 80 °C was found to be appropriate and adequate (~99%) to release sialic acid from homopolymeric group C polysaccharide when we studied extensively the sialic acid peak contributions from single or in different combinations of four serogroup polysaccharides. This condition may not suffice to either W or Y (disaccharide repeat polysaccharides) hydrolyses to release total sialic acid. Peaks at different retention times other than S1 and S2 may be a result of partially truncated species with differential charges. At least one of these were common (e.g., SA_2) to both W and Y, and some species (SA 3 and 4) are distinctly different for W and Y, respectively. The peak retention time difference among W and Y could be due to the charge difference attributed to neutral sugar that was either α 2 → 6 linked galactose or glucose in the source polysaccharide structure still not completely removed during mild hydrolysis.

CONCLUSIONS

In this article, a method of quantification of each polysaccharide in a meningococcal tetravalent A/C/Y/W serogroup polysaccharide individually conjugated to diphtheria toxoid protein (A/C/Y/W-135-DT) conjugate vaccine is described. This method relies on making individual serogroup standard polysaccharide calibration curves, which are then used to quantify the unknown microgram quantities of final bulk or vial PSs. The mild hydrolysis results of sialic acid containing polysaccharides (C, W, and Y) showed only a C polysaccharide; a homopolymer of sialic acid undergoes near-complete hydrolysis, whereas W or Y undergo partial hydrolysis. Quantifying W or Y based on subtraction methods in a mixture would give ambiguous results.

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Notes

The authors declare no competing financial interest.

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