NMR Assays for Estimating the O-Acetyl Content of Meningococcal Polysaccharide Serogroup A in Quadrivalent Conjugate Vaccine Formulation

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ABSTRACT: The use of multivalent glycoconjugate vaccines has dramatically contributed to reduce the incidence of meningococcal infectious disease. The advanced structural characterization of polysaccharide conjugates leads to enhancements in the quality and control of the products. Here, we report a novel nuclear magnetic resonance (NMR) method to confirm the identity and structural conformity (e.g., O-acetyl content) of saccharide antigens that comprise a licensed tetravalent meningococcal serogroups A, C, W, and Y vaccine. For the first time, the NMR methodology is applied on a formulation (licensed vaccine) containing a large excess of excipient (i.e., sucrose) without analytical sample pretreatment. This work confirms the applicability of a rapid and easy NMR assay on a multivalent conjugate vaccine, which might be extended to other combination vaccines that are already licensed or in clinical development.

INTRODUCTION

Multivalent polysaccharide (PS) conjugate vaccines have been licensed to prevent bacterial infection caused by serogroup A, C, W, and Y of Neisseria meningitidis.1 A pentavalent combination vaccine including the additional serogroup X is currently under clinical development and the results of a phase I clinical trial have been recently published.2 When different antigens are combined to form a multivalent vaccine, a panel of tests that is able to characterize the formulation in terms of potency, stability, identity, and strength has to be developed. Although tests might be in place for single components, it is important to determine if their combination gives rise to compatibility issues that might change some characteristics of single antigens (i.e., stability, immunogenicity). Physicochemical and immunological tests are commonly used to compare the biological activity of individual antigens before and after the combination, allowing for the quantification of any chemical/immunological interference. The structural integrity and stability of PS-based antigens is a critical quality attribute to be assessed after combination and is a common indicator of the product stability and vaccine potency. For instance, the generation of the unconjugated saccharide or the release of functional groups (i.e., O-acetylation, OAc) relevant for a functional PS epitope can dramatically affect the vaccine potency.

The meningococcal (Men) serogroups A, C, W, and Y capsular PS repeating units have the chemical structures →6)-α-D-ManpNAc(3/4OAc)-(1→OPO3→; →9)-α-D-Neu5Ac(7/8OAc)-(2→; →6)-α-D-Galp-(1→4)-α-D-Neu5Ac(7/9OAc)-(2→; →6)-α-D-GlcP-(1→4)-α-D-Neu5Ac(7/9OAc)-(2→, respectively (Figure 1).3 MenA PS is partly O-acetylated at position C3 or C4, while all MenCWY PS contain Neu5Ac moiety in the relative repeating units that are partially O-acetylated at the glycerol chain of Neu5Ac (at position C7 and C8 for serogroup C and at position C7 and C9 for serogroup W and Y).3

Nuclear magnetic resonance (NMR) spectroscopy is currently used in the advanced characterization and quality control of existing and novel products.4,5 NMR applications have been first developed and implemented for structure determination and for identity/integrity testing of the carbohydrate components. NMR provides a single generic tool that is able to replace a wide range of wet chemical/
Herein, we report the development of a novel NMR-based assay applied to the tetravalent vaccine against meningococcal serogroup A, C, W, Y infection, licensed for human use in a two-vial presentation (a lyophilized monovalent MenA component with sucrose/potassium dihydrogen phosphate as excipient to be reconstituted with the liquid MenCWY component immediately before injection), and an investigational liquid formulation currently in clinical trial. 

The commercial Menvac vaccine contains size-reduced oligosaccharides of serogroups A, C, W, and Y, conjugated to a nontoxic mutant of Corynebacterium diphtheriae toxin, termed “cross reacting material 197” (CRM197). Each 0.5 mL dose of vaccine contains 10 μg of MenA oligosaccharide and 5 μg of each of MenC, MenY, and MenW oligosaccharides and 32.7–64.1 μg of CRM197 protein.

This methodology has confirmed the applicability of NMR spectroscopy on a final combination conjugate vaccine as an identity and structural conformity test (i.e., O-acetyl content of the MenA antigen) of the carbohydrate moieties.

# RESULTS AND DISCUSSION

Identity Test of MenACWY Antigens. In accordance with the strategy applied for the development of an identity test method successfully developed for the 23-valent pneumococcal PS-based combination vaccine (Pneumovax23, Merck), where one proton NMR signal as the “probe signal” for each antigen has to be compared with the reference and also with the experience collected internally on the method developed for MenACWY monovalent purified PS antigens; other process intermediates and bulk glycoconjugates (recently included as the reference method in the USP monograph (198)), where five signals for each antigen have been used for comparison with references, the feasibility of a new identity test has been investigated.

As shown in Figure 1 (schematic representation of MenACWY-CRM197 conjugates), the proton signals H_{ManNAc} (proton at position C1 of ManNAc residues) for MenA, H_{Neu5Ac-8OAc} (proton at position equatorial C3 of the Neu5Ac residues O-acetylated at position C7) for MenC, H_{Neu5Ac-7OAc} (proton at position C1 of the Gal residues) for MenW, and H_{Neu5Ac-8OAc} (proton at position C1 of the Glc residues) for MenY have been selected as probe signals. For MenC, the protons at C1 equatorial position of Neu5Ac residues resonate at different chemical shifts, depending on the position (C5 or C6) of the OAc group (2.92 and 2.79 ppm for H_{Neu5Ac-8OAc} and H_{Neu5Ac-7OAc} respectively). As published in the literature, the H_{Neu5Ac-7OAc} signal includes both the OAc residues flanked at their reducing side by an OAc residue and by a 7OAc residue. Because H_{Neu5Ac-7OAc} overlaps with the H_{Neu5Ac-8OAc} signals of MenW and MenY, the H_{Neu5Ac-8OAc} peak has been selected for identity of MenC.

The 1H NMR profile of Menvac vaccine, acquired by using a diffusion filter pulse sequence to suppress the sucrose signals (Figure 2), shows the peaks of the four antigens used as identity probe signals for MenA, MenC, MenW, and MenY antigens (Table 1).

Quantification of O-Acetyl Content of the MenA Antigen. The quantification of the OAc level allows for the evaluation of the PS antigen conformity, which may also occur because of adverse events which could provoke the release of O-acetyl groups. The MenA plain PS is largely O-acetylated at position C4 (>90%) and slightly at position C3 (Figure 1). The importance of OAc in MenA antigen immunogenicity has
been confirmed in preclinical study. Specific antibodies elicited in post-immunization human sera mostly identified O-acetylated PS residues impacting the degree of antibody inhibition. Comparative immunogenicity studies in mice revealed that de-O-acetylated antigens resulted in a marked loss of immunogenicity (total IgG) and, most relevantly, in their ability to induce functional bactericidal antibodies. In a phase III partially blinded, controlled study [i.e., 1170 healthy subjects aged 18–25 years were randomized (1:1:1)], two lots of meningococcal ACWY-tetanus toxoid conjugate vaccine (MenACWY-TT) that differed in serogroup A PS OAc levels (68% vs 92% OAc), respectively, were evaluated in terms of immunogenicity (i.e., rabbit serum bactericidal activity) and safety in comparison to a licensed MenACWY PS vaccine (82% serogroup A OAc). The MenA-TT conjugates with the OAc level of 68 and 92% resulted in comparable vaccine immunogenicity, considering that, however, the vaccine population was seropositive at the time of vaccination. As a matter of fact, all the meningococcal serogroup A conjugates contained in licensed vaccines (MenAfriVac—Serum Indian Institute; Menveo—GSK Vaccines; Menactra—Sanofi Pasteur; Nimenrix—GSK Vaccines later taken over by Pfizer) are largely O-acetylated. However, no evidence has been collected to define the minimal OAc level affecting the immunogenicity of meningococcal serogroup A vaccines so far.

High relevance of O-acetyl moieties of MenA PS could also be related to the presence of this decorative group within the saccharide ring, probably in a unique arrangement that could not be easily mimicked in its absence.

For the monovalent MenA PS and conjugate, the OAc content can be estimated by integration of the H2 ManNAc-3OAc peak (proton at position C2 of ManNAc residues O-acetylated at C-3), H2 ManNAc-4OAc peak (proton at position C2 of ManNAc residues O-acetylated at C4), and H2 ManNAc-deOAc peak (proton at position C2 of ManNAc residues without OAc), in comparison to H1 ManNAc (proton at position C1 of ManNAc residues). The total OAc level can be obtained by the sum of H2 ManNAc-3OAc and H2 ManNAc-4OAc peak integration as well as by integrating the HManNAc-3/4OAc peak. The same method already developed for the monovalent MenA conjugate cannot be applied to the MenACWY combination vaccine as the NMR profile exhibits large overlapping signals because of the presence of the four antigens (Figure 2). However, the HManNAc peak at approximately 5.5 ppm, which appears as a complex peak, is not affected by the presence of other antigens. As reported in Figure 3a, the 1H–31P heteronuclear multiple-bond correlation (HMBC) NMR spectrum provides a clear assignment of different MenA residues, depending on different OAc patterns, terminal residues, and so forth.

The HManNAc nuclear spin resonates at a slightly different frequency and correlates with the diester phosphate group (the monoester phosphate signal falls at higher chemical shift15) depending from the specific magnetic environment generated by (a) presence of the OAc group at position C3 or C4 (b) geminal with the di-ester phosphate group of the open ring unit at reducing end terminus (unit #2) (see Figure 1). The HMBC spectrum shows six 1H signals (excluding the satellite peaks), originated by the scalar long–range correlation (H–C–O–P) with diester 31P signals (separated peaks for OAc, −5.2 ppm, and non OAc, −5.7 ppm, forms). As shown in Figure 3a, the proton at position C1 of several ManNAc species O-acetylated at position C1 (3OAc) or C4 (4OAc) or non-O-acetylated (deOAc) resonate at different chemical shift values. From downfield to upfield, the signals H2 3OAc, H2 3OAc, H2 4OAc/H2 3OAc, H2 4OAc/H2 3OAc, H2 4OAc/H2 4OAc resonate in the sequence and some signals partially overlap each other.

As shown in Figure 3b, integral areas have been defined according to the assignment of different HManNAc species and also considering “operative” reasons (i.e., the presence of a valley at ~5.46 ppm) related to the overall peak shape. In essence, the OAc content has been calculated according to the formula % OAc = H1 4OAc/H1 × 100, where H1 (range 5.52–5.34 ppm) is the sum of H1 4OAc (range 5.52–5.46 ppm) and H1 deOAc (range 5.46–5.34 ppm) peak integrals. Because the integration is performed in a restricted spectral window (5.52–5.34 ppm), the noise baseline does not affect the integral values significantly.

As compared to the OAc content measured on the monovalent MenA bulk conjugate by integrating the signals of protons at C2 (H2 3OAc, H2 4OAc, H2 deOAc) and the protons at C3/C4 positions (H3 3OAc + H4 3OAc) in comparison to protons at the C1 position, the method applied by dissecting the HManNAc provides a lower estimation (Table 2).

Because of the partial overlapping of the H1 signals of OAc and non OAc forms, the integration strategy in the one-dimensional spectrum results in under estimation of approximately 10% of OAc content for the OAc level in the range of 70–80%. Essentially, some of the OAc species are

| Table 1. Chemical Shift (δ) of HManNAc, HNeuAc, HGal, MenW, HManY Peaks Used as Identity Probe Signals for MenA, MenC, MenW, and MenY Antigens, Respectively |
|---------------------------------------------------------|
| MenA | MenC | MenW | MenY |
| δ (ppm) | ~5.46 | ~2.79 | ~5.11 | ~5.08 |

| Table 2. % OAc Estimated on a Monovalent MenA-CRM197 Sample by Integrating Different Signals: (a) % OAc = H1 4OAc/H1 × 100, Where H1 is the Sum of H1 4OAc and H1 deOAc Peak Integrals; (b) % OAc = (H3 3OAc + H4 3OAc)/H1 × 100, Where H1 is the Sum of H1 4OAc, H1 3OAc, and H1 deOAc Peak Integrals; (c) % OAc = (H1 4OAc + H1 3OAc)/H1 × 100, Where H1 is the Sum of H1 4OAc and H1 3OAc Peak Integrals (Figure 4) |
|-------------------|-----------|-----------|-----------|
| % OAc | 66 | 78 | 80 |
grouped with the non OAc form in the integral measurement (Figure 3).

**CONCLUSIONS**

Novel applications for the identity test and O-acetyl content estimation of the MenA antigen in multivalent MenACWY combination vaccines have been developed.

NMR-based identity assay confirmed very high specificity and reproducibility and should be considered as a suitable alternative to commonly used non-specific and time-consuming colorimetric/chromatographic assays.

This NMR methodology can be readily adapted for use with other bacterial PS preparations. For instance, it has the potential to be applied on other licensed mono- and multivalent conjugate vaccines against *Haemophilus influenzae* type b (PedvaxHIB—Merck; ActHIB—Sanoﬁ Aventis Pasteur; Hiberix—GSK Vaccines), meningococcal (Menjugate, Menitorix—GSK Vaccines; Menactra—Sanoﬁ Pasteur; NeisVac-C, Nimenrix—Pﬁzer; Meningitec—Nuron Biotech; MenAfriVac—Serum Indian Institute), meningococcal/ *H. influenzae* type b (MenHibrix—GSK Vaccines), and pneumococcal (Prevnar, Prevnar13—Pﬁzer; Synflorix—GSK Vaccines) infections.

A new method for the estimation of OAc content of the MenA antigen in Menveo-licensed vaccine, based on the integration of $H_1$ signals in one-dimensional spectra, has been developed.
adopted for use with other vaccine preparations potentially including protein antigens.

**MATERIALS AND METHODS**

The Menveo vaccine, as a two-vial presentation (monovalent lyophilized MenA-CRM197 and trivalent liquid MenC-, MenW-, and MenY-CRM197) as obtained from GSK Vaccines Manufacturing.

**NMR Samples Preparations.** To prepare NMR analytical samples, 10 vials of lyophilized MenA-CRM197 were reconstituted with 10 vials of liquid MenCWY singularly. All the them were pooled in 8 mL glass vial (Wheaton) and finally dried under vacuum.

A monovalent MenA-CRM197 sample was also prepared by drying under vacuum approximately 1 mg of conjugate as the saccharide content.

The lyophilized contents were solubilized in 0.6 mL of deuterium oxide (99.9 atom % deuterium; Aldrich), mixed to obtain a uniform concentration, and subsequently transferred to a 5 mm NMR tube (Bruker).

**NMR Measurements.** Proton NMR experiments were recorded on a Bruker Aeon AVANCE III 600 MHz spectrometer equipped with a high-precision temperature controller using a 5 mm QCI CryoProbe (Bruker). TopSpin software (Bruker) was used for data acquisition and processing. 1H NMR one dimensional spectra were collected at 600 MHz and 25 ± 0.1 °C, with 32k data points over a 12 ppm spectral width, accumulating a sufficient number of scans. The transmitter was set at the water frequency, which was used as reference signal (4.79 ppm).

To suppress the sucrose signals, which dominate the spectrum because of its large excess (i.e., >1500 molar fold) in comparison to the MenACWY antigens, a diffusion filter pulse sequence (ledpgp2s1d) was applied by following an appropriate setting of the acquisition parameters (i.e., gradient pulses, delays). The spectra were weighted with 2.0 Hz line broadening and Fourier-transformed.

31P NMR one-dimensional spectra were recorded at 161.9 MHz at 25 ± 0.1 °C, with 32k data points over a 20 ppm spectral width, accumulating an appropriate number of scans. The spectra were weighted with 3.0 Hz line broadening and Fourier-transformed. Phosphoric acid (85%) in deuterium oxide was used as external standard (0 ppm).

All the 1H and 31P NMR spectra were obtained in a quantitative manner by using a total recycle time to ensure a full recovery of each signal (5 × longitudinal relaxation time T1).

The 1H–31P two-dimensional HMBC NMR experiment was recorded on the monovalent MenA-CRM197 sample by a standard pulse program. 4096 and 512 data points were collected in F2 and F1 dimension, respectively. A sufficient number of scans was accumulated prior to Fourier transformation to yield a digital resolution of 0.2 and 3.0 Hz per point in F2 and F1, respectively.

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**Author Contributions**

The manuscript was written through contributions of all the authors. All the authors have given approval to the final version of the manuscript.

**Notes**

The authors declare the following competing financial interest(s): All the authors are employees of the GSK companies. Francesco Berti (corresponding author) is owner of patents on related topics. This study was sponsored by GSK Biologicals SA.

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