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Use and validation of NMR assays for the identity and *O*-acetyl content of capsular polysaccharides from *Neisseria meningitidis* used in vaccine manufacture

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Abstract

We describe a validated NMR (nuclear magnetic resonance) spectroscopic assay for the identity of the capsular polysaccharides (CPSs) from *Neisseria meningitidis* Groups A, C, W135 and Y used in vaccine manufacture, and to determine the proportion of residues carrying an *O*-acetyl substituent. Proof of structural identity and quantitation of the *O*-acetyl content are key control parameters for these vaccines. The meningococcal CPSs have variable levels of *O*-acetylation, present at multiple sites in the repeat unit, leading to complex NMR spectra. Base-catalysed de-*O*-acetylation of the Groups A, C, W135 and Y CPSs yields simplified and reproducible spectra suitable for comparison with reference data. The degree of *O*-acetylation of the original CPS can be determined by integration of the acetate anion resonance and a suitable resonance from the saccharide moiety. The assay was validated using 46 independent samples from five manufacturers, and is shown to be robust and reproducible.

Keywords: *Neisseria meningitidis*; Meningococcal polysaccharides; Glycoconjugate vaccine; NMR spectroscopy

1. Introduction

Meningococcal meningitis remains a feared disease, due its prevalence in infants and young adults, its rapid progression from relatively mild early symptoms, the continuing high death rate, and the severe neurological damage that can be inflicted on survivors. The World Health Organisation (WHO) estimated that there are approxi-

Abbreviations: CPS, capsular polysaccharide; Hib, *Haemophilus influenzae* type b; Men A, meningococcal Group A CPS; Men C, meningococcal Group C CPS; Men B, meningococcal Group B CPS; Men Y, meningococcal Group Y CPS; Men W135, meningococcal Group W135 CPS; Men BPr, *N*- propionylated analogue of the meningococcal Group B CPS; NMR, nuclear magnetic resonance spectroscopy.

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Group A	→6)-D-Man*p*NAc(3/4OAc)-α-(1→OPO_3→	Degree of *O*-acetylation ca. 90 %
Group B	→8)-D-Neu*p*5Ac-α-(2→	Not *O*-acetylated
Group C	→9)-D-Neu*p*5Ac(7/8OAc)-α-(2→	Degree of *O*-acetylation close to 100 %
Group Y	→4)-D-Neu*p*5Ac(7/9OAc)-α-(2→6)-D-Glc-α-(1→	*O*-acetylated
Group W135	→4)-D-Neu*p*5Ac(7/9OAc)-α-(2→6)-D-Gal-α-(1→	*O*-acetylated
N-Propionylated Men B	→8)-D-Neu*p*5Pr-α-(2→	Not O-acetylated.

Fig. 1. Structure of the repeat units of the CPSs of the five meningococcal serogroups commonly associated with disease, and the structurally-modified Group B CPS proposed for use in a conjugate vaccine.

mately 500 000 cases of meningococcal meningitis annually, with 50 000 deaths and 60 000 survivors suffering severe neurological sequelae [1]. The death rate for meningococcal septicaemia remains 18% even in developed countries [2]. Twelve serogroups of *Neisseria meningitidis* are known [3], which differ in the structure of their capsular polysaccharides (CPSs). Of these, Group A is responsible for epidemic meningitis in sub-Saharan Africa, whilst Groups B and C cause endemic in developed countries. Whilst Groups Y and W135 have traditionally been associated with low levels of disease, often in patients with complement deficiencies, the incidence of Group Y disease in the US has increased to 25% [4], and Group W135 was associated with a recent outbreak following the 2000 Hajj pilgrimage [5]. The other serogroups are very rarely associated with disease, and, to the best our knowledge, are not targets for vaccine development. The incidence of meningococcal disease appears to be increasing: in the UK the number of laboratory-confirmed cases of meningococcal disease (both Groups B and C) almost doubled between 1989 and 1999 [6]. The structures of the repeat units of these CPSs are shown in Fig. 1.

Although meningococcal vaccines consisting of mixtures of purified CPSs have been in use for a number of years, their use has been restricted because they are not effective in infants (a major risk group) and give relatively short duration of protection. Current vaccines are either bivalent (containing Groups A and C CPSs—Mengivac (A+C) and AC VAX) or tetravalent (containing CPSs of Groups A, C, Y, and W135—ACWY VAX, Menomune® and Tetramune®) [2]. Recently, glycoconjugate vaccines containing oligosaccharides derived from the Group C CPS covalently attached to either CRM197 (since 1999) or tetanus toxoid (since 2000) have been available in the UK, and were introduced into mass paediatric and catch-up vaccination campaigns. Early data suggests that these vaccines are highly effective in preventing disease [7]. Glycoconjugate vaccines employing the Group A [8,9] and Groups Y and W135 CPSs [10] are also in development. The Group B CPS is poorly immunogenic, and many subtle approaches to make effective Group B vaccines have been tested, including preparation of glycoconjugates utilising *N*-propionylated Men B CPS [11–14]. A vaccine of this kind is expected to enter clinical trials in the near future.

A key control test both for CPS and glycoconjugate vaccines is to establish the identity of the saccharide component. Traditionally this has been accomplished using a combination of wet chemical methods and an immunological identity test [15]. For all four meningococcal CPSs, there is also a minimum level of *O*-acetylation specified, usually determined by means of the Hestrin assay to quantify the millimoles of *O*-acetyl groups per

Table 1
Pharmacopoeial specifications for meningococcal CPS used in vaccine production

Serogroup	**Protein (%)**	**Nucleic acid (%)**	***O*-acetyl (mmol/g)**	***O*-acetyl % of repeats[a]**	**Phosphorus (mg/g)**	% Purity phosphorus	**Sialic acid (mg/g)**	% Purity sialic acid	**% > Kd = 0.5**
A	< 1	< 1	> 2	> 61.5	> 80	> 79	–	–	> 65
C	< 1	< 1	> 1.5	> 50	–	–	> 800	> 86	> 75
Y	< 1	< 1	> 0.3	> 14.3	–	–	> 560	> 87	> 80
W135	< 1	< 1	> 0.3	> 14.3	–	–	> 560	> 87	> 80

Columns where the heading is in bold are pharmacopoeial specifications: other columns giving 'purity' or "% of repeats' values are figures derived from these. Calculations are based on the CPS being present as the monosodium salt of each repeat unit. There are no specifications for the Group B CPS or its *N*-propionylated form.

[a] Assumes pure CPS, figures increase to 54% (Men C), 16% (Men Y) and 16% (Men W135) for polysaccharide of minimum purity.

gram dry weight of polysaccharide (Table 1): this approach demands access to relatively large amounts of material. Recently, we and others have used NMR (nuclear magnetic resonance) spectroscopy to control the quality of bacterial CPSs used in vaccine production, in particular those from *Haemophilus influenzae* type b (Hib) [16,17], *Streptococcus pneumoniae* [18], and the *Salmonella typhi* Vi CPS [19]. These studies have been supported by an ongoing effort to confirm the structures of the CPSs and to achieve full NMR assignments [20]. NMR has also proven extremely valuable in monitoring production of glycoconjugate vaccine [21] and in monitoring the degradation of the saccharide components of these vaccines [22–25].

Whilst the structures of these CPSs were some of the first to be established by NMR spectroscopy [26–29], full ^{1}H NMR assignments and detailed *O*-acetylation patterns have been reported for the clinically relevant meningococcal CPSs [20,30–32]. The presence of variable degrees of *O*-acetylation spread over multiple sites, and the changes in *O*-acetylation pattern which occurs with time [20], makes validation of a simple test, in which identity is assigned by comparison of the spectrum of a test sample with a reference spectrum, problematic. We have therefore adopted an approach developed for the *S. typhi* Vi CPS [19]: the native CPS in the NMR tube is de-*O*-acetylated by addition of sodium deuteroxide, and spectra obtained for both the native and the de-*O*-acetylated material. The spectrum of the CPS is simplified by de-*O*-acetylation and, by integrating appropriate resonances, the degree of *O*-acetylation of the original CPS can be determined. Whilst there is a growing body of evidence that *O*-acetylation is not important to elicit a protective antibody response, for Group C [33–36] and Group W135 [37], it does appear to be important for Group A [38]. A recent study [39] showed that only 8% of Men W135 and 79% of Men Y isolates in the UK expressed *O*-acetylated capsules. Glycoconjugate vaccines using de-*O*-acetylated Group C saccharide chains are licensed in the UK, and we show that the same protocol can be used as part of the quality assurance regime of such material.

2. Materials and methods

2.1. Standard experimental conditions

NMR spectra were acquired on a Varian Unity 500 NMR spectrometer equipped with a 5 mm PFG triple resonance probe, high precision temperature controller (±0.1 °C), and under the control of VNMR version 6.1B, or a Varian Inova 500 spectrometer equipped with a 5 mm PFG inverse detection heteronuclear probe, running under VNMR version 6.1C and Solaris 2.8. Weighed samples (typically 2–3 mg) of the polysaccharide are dissolved in deuterated water (700 μl: M&G Chemicals, Glossop) for analysis. De-*O*-acetylated samples were prepared by addition of 40% sodium deuteroxide in deuterated water (Goss Scientific Instruments, UK: 15 μl into 700 μl, corresponding to a final concentration of ca. 200 mM) to the sample in the NMR tube. For every test sample, two spectra are collected: the first that of the native polysaccharide and second soon after de-*O*-acetylation. Standard spectral acquisition conditions are to collect 64 k data points over a spectral window of 8000 Hz. The acquisition time is 4.096 s and a relaxation delay of 26 s is included, giving a recycle time of 30 s. Typically 80 scans are averaged (giving a total acquisition time of approximately 40 min), although more scans may be collected if less sample is available. Spectra were Fourier-transformed after applying a 0.2 Hz line broadening function and referenced relative to the acetate anion resonance at 1.91 ppm or added TSP-d_4 at zero ppm or −1.8 ppm (^{13}C) [40], both under neutral conditions and in 200 mM NaOD. The baseline is corrected by the spline method using standard VNMR software: spectral regions in which baselines are forced are indicated in the relevant figures. Integrations were performed using subroutines built into the VNMR software. The integration limits were from 2.25 to 1.96 ppm for the *N*-acetyl resonance and from 1.96 to 1.86 ppm for the acetate anion methyl resonance. To maximise the data available from limited sample and spectrometer time, some spectra were obtained using 5 mm susceptibility-matched NMR tubes (Shigemi, Tokyo), containing the CPS sample in 350 μl of deuterated water.

2.2. Samples

CPS samples were provided by vaccine manufacturers, and were either material provided for batch release purposes, for pre-licensing evaluation, or were generous gifts of development materials. Neutral samples of the de-*O*-acetylated CPS were obtained by desalting of combined samples de-*O*-acetylated in the NMR tube. Typically, three samples were combined and passed through a column of BioRad AG1 × 8, 100–200 mesh H^+ form (1 ml bed volume), and freeze dried. The samples were passed through a C18 SepPak (to remove some coloration from the ion-exchange resin), eluted with water/5% ethanol and deuterium-exchanged by repeated lyophilisation prior to NMR analysis. The de-*O*-acetylated Men C CPS and the periodate oxidation-derived oligosaccharide was material provided by Baxter Hyland Immuno for batch release purposes, and was a sample of material intended for vaccine production.

2.3. Validation experiments

Proton and ^{13}C NMR spectra were assigned at 30 °C and 50 °C using conventional 2D techniques, including double-quantum filtered COSY, TOCSY and HSQC spectra and by comparison with published data. Spectra were obtained on de-*O*-acetylated samples at neutral/slightly acidic pH (see above) or on in situ de-*O*-acetylated material in 50, 100, 200 or 400 mM NaOD at an indicated probe temperature of 30 °C. Spectra on samples in 200 mM NaOD were obtained at 30 and 50 °C, simulating a failure of the cold junction of the temperature controller of our spectrometer. Typically ca. 2 mg of CPS was used, but spectra were obtained using between 0.5 and 5 mg of sample (Groups W135 and Y only). Coefficients of varia-

tion were determined using five samples of a single polysaccharide bulk, and for each CPS. Statistical analysis was performed in an Excel spreadsheet using inbuilt functions.

3. Results and discussion

NMR spectroscopy has proven a structurally sensitive and reproducible technique to control the identity of bacterial polysaccharides used in vaccine manufacture [16–19], but is complicated by the presence of incomplete or labile *O*-acetylation, when samples may be derivatised to different extents. We have reported ^{1}H NMR assignments for the native *O*-acetylated Men C CPS, and shown that *O*-acetyl group migration [20] complicates implementation of a simple NMR identity test. An approach using base-catalysed de-*O*-acetylation in situ was implemented, and the initial conditions chosen were those used for the *S. typhi* Vi CPS [19], apart from the sample temperature. Briefly, the CPS was dissolved in deuterated water, and an initial spectrum collected to provide information on purity and the acetate content of the sample. A volume of sodium deuteroxide (40% in deuterated water, approximately 10 M) was then added to give a final base concentration of approximately 200 mM, the sample left until de-*O*-acetylation is complete, and the NMR spectrum recorded again. The *O*-acetyl content of the original sample was determined from the relative integrals of the acetate resonance and a resonance arising from the saccharide chain. Correction for the acetate present in the original sample is possible if necessary. Validation experiments were designed to show these conditions are appropriate, and that the assay is robust and reproducible.

3.1. NMR spectra of the native CPS samples

The 500 MHz ^{1}H NMR spectra of the native, *O*-acetylated CPS matched very closely those reported previously [20]. Material of the same serogroup from different manufacturers all appeared similar, but variable *O*-acetylation was apparent, although the location of *O*-acetyl substituents was the same in material from different manufacturers. In most samples broad, low intensity resonances were apparent from impurities, thought to include residual antifoam agents and hexadecyl trimethyl ammonium bromide (Cetavalon). Minor resonances from acetate anion were also observed (see below).

3.2. De-*O*-acetylation of the CPSs

De-*O*-acetylation of the Groups A, W135 and Y CPSs in 200 mM NaOD at room temperature was rapid and complete by the time the sample was re-analysed, typically 20 min after NaOD addition. De-*O*-acetylation of the Group C CPS was slower, and samples were incubated (typically 37 °C, 1 h) prior to NMR analysis. This is consistent with our experience with the *S. typhi* Vi CPS [19], which also showed that with a 50 mM final base concentration the progress of de-*O*-acetylation could be followed in the spectrometer. As found for *S. typhi* Vi CPS, narrowing of the resonances occurred when the samples were treated with base. For examples, the half-height line-width ($\Delta\nu_{1/2}$) of the α-Glc anomeric resonance in the Group W135 was found to be reduced from about 11 to 9.5 Hz after addition of base. Inter-proton coupling constants were resolved in de-*O*-Ac Men C and Men A, and in the latter very high quality spectra were obtained (Fig. 2). In some samples a slight opalescence appeared after NaOD addition, thought to be due to the CPS being present as a calcium salt. This did not appear to influence the quality of the spectra obtained or the quantitation of the *O*-acetyl content.

3.3. Specificity of the test method

The ^{1}H spectra from all the de-*O*-acetylated serogroups are quite distinct so that identity can be achieved by visual inspection. These spectra are also quite distinct, by visual inspection, to all other bacterial CPSs used in vaccine manufacture (data not shown). For example, two structurally related

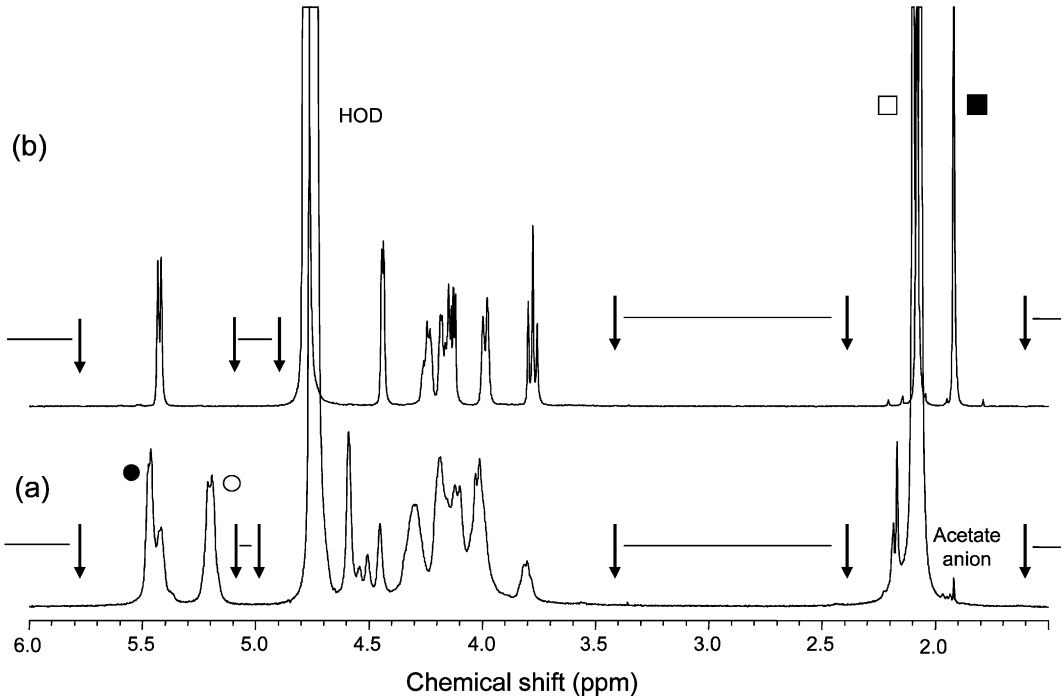

Fig. 2. Partial 500 MHz ^{1}H NMR spectra at 30 °C of (a) Native *O*-acetylated Men A CPS under neutral conditions, and (b) the same sample after addition of NaOD to a final concentration of 200 mM. F.i.d.s were weighted with 0.2 Hz line broadening and baseline corrected by application of a spline function, forcing baselines in those spectra regions indicated by the horizontal bar. The *O*-acetyl content of the native CPS was estimated from the relative integrals of the overlapping (ManNAc(3OAc) H-3 and ManNAc(4OAc) H-4) resonances (marked with an open circle) and the ManNAc H-1 (marked with a filled circle). The *O*-acetyl content of the native CPS was estimated from the base de-*O*-acetylated CPS by comparison of the integrals of the ManNAc *N*-acetyl methyl (marked with an open square) and the acetate anion (marked with a filled square). The acetate content of the native CPS at 1.908 ppm, labelled, was estimated at ca. 1% and not corrected for in the subsequent quantitative analyses. Vertical arrows separated by horizontal lines indicate regions in which the baseline was forced to zero prior to integration.

pairs can be readily compared: Groups W135 and Y, which differ in the configuration at the hexose C-4 (Glc or Gal), and the de-*O*-Ac Men C and native Men B, which differ in their inter-residue linkage (α2→8 for Men B, α2→9 for Men C). The spectra of the W135 and Y serogroups are compared in Fig. 3. The chemical shifts of the hexose H-1 differ by 0.035 ppm and of the *N*-acetyl resonance by 0.058 ppm. The shape of the envelope of resonances between 3.45 and 4.2 ppm differs markedly, largely due the presence of more highfield resonances from the Glc residue in Men Y. Similarly the ^{1}H spectra of the de-*O*-acetylated Men C and Men B, either under neutral conditions or in 200 mM NaOD (Fig. 4) are quite distinct, with lowfield resonances from the H-8 and H-9 diagnostic of Men B, and differences in the chemical shifts of nearly all the resonances, including the Neu5Ac H-3a, H-3e and *N*-acetyl methyl, apparent.

3.4. Assignment of endgroup resonances and determination of the average chain length of oligosaccharides derived by periodate oxidation of de-O-acetylated Men C CPS

The average chain length of these oligosaccharides can be estimated, in the same NMR experi-

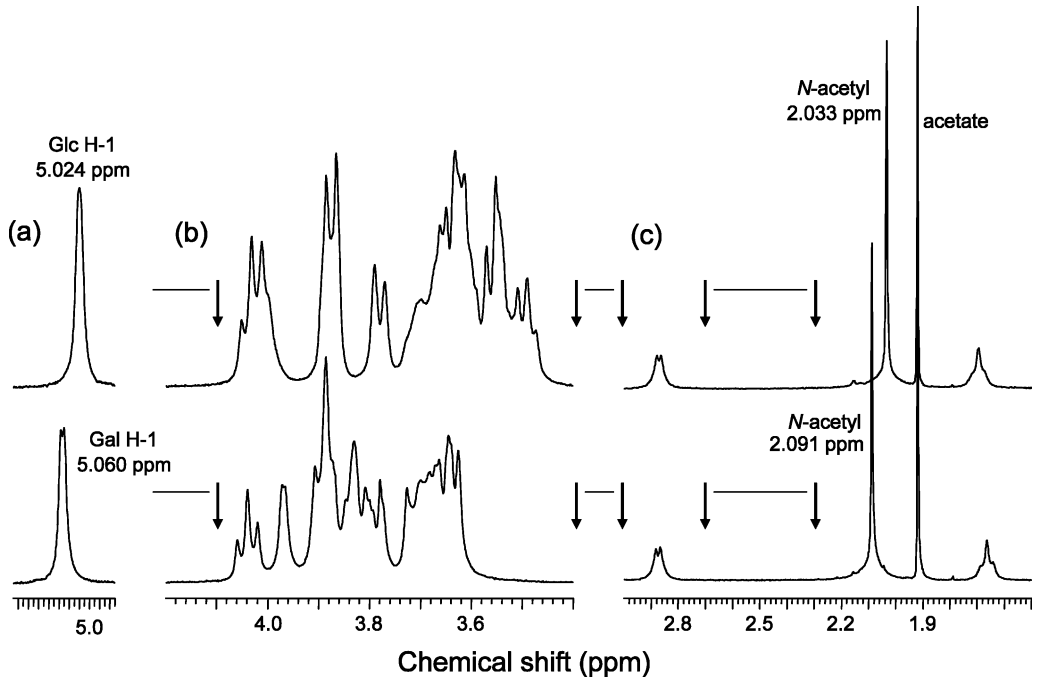

Fig. 3. Comparison of partial 500 MHz ^{1}H NMR spectra of the de-*O*-acetylated Group W135 (bottom) and Group Y (top). Spectral regions diagnostic of the serogroup are highlighted: (a) the chemical shift of the hexose H-1, (b) the envelope of resonances between 3.45 and 4.2 ppm, and (c) the differences in chemical shift of the Neu5Ac NAc methyl resonance. Spectra were obtained at 30 °C on samples in 200 mM NaOD. Vertical arrows separated by horizontal lines indicate regions in which the baseline was forced to zero prior to integration.

ment as used to confirm identity, by integration of resolved resonances from the novel endgroups formed during periodate oxidation, compared to the those of internal residues. This approach has been applied for oligosaccharides derived from the Hib PRP [41], but assumes that all the end groups are identified (e.g. both hydrated and non-hydrated aldehydic functions) and assigned, and all relevant resonances included in the determination. The assignments are listed in Table 2, and the spectrum of an oligosaccharide derived by periodate oxidation of de-*O*-acetylated Men C CPS is shown in Fig. 5. The chemical shifts of internal Neu5Ac residues in the oligosaccharide are nearly identical to those in the polysaccharide. Three resonances of low but equal intensity are well resolved, and were assigned by correlation methods as 'reducing terminal' glycolaldehyde residue and the H-7 of the 'non-reducing' terminal Neu5Ac fragment. The Neu5Ac H-3e and NAc resonances have low intensity peaks overlapping at high field, thought to arise from $\rightarrow$9Neu5Acα2$\rightarrow$ $OCH_2CH(OH)_2$ and the 'non-reducing terminal' Neu5Ac fragment, respectively. No minor resonances were detected at low field, consistent with the aldehydic groups being completely hydrated.

3.5. Assignment of the NMR spectrum of the de-*O*-acetylated CPSs

Proton and ^{13}C spectra were assigned for samples in 200 mM NaOD at 30 and 50 °C, using conventional homo- and hetero-nuclear methods. The data are collected in Table 3.

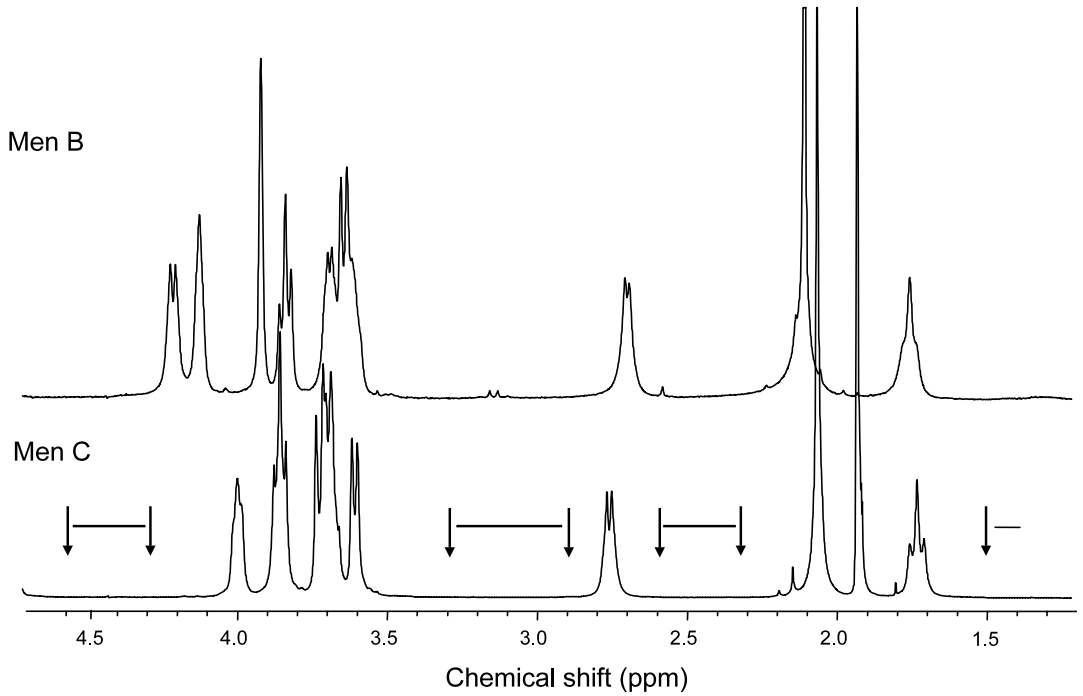

Fig. 4. Comparison of partial 500 MHz ^{1}H NMR spectra of the Men B (top) and de-*O*-Ac Men C CPSs (bottom), highlighting features diagnostic of the serogroup. Spectra were obtained at 30 °C on samples in 200 mM NaOD. Vertical arrows separated by horizontal lines indicate regions in which the baseline was forced to zero prior to integration.

Table 2
Chemical shift assignments for oligosaccharides derived by de-*O*-acetylation and periodate oxidation

Residue	H-3a/H-3a C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-7/H-7′ C-7	H-8/H-8′ C-8	H-9/H-9′ C-9	NAc
Oxidised material[a]								
Residue A				3.747	4.929 88.20			
Internal NeuNAc	1.713/2.740 39.58	3.654 68.05	3.840 51.25	3.977 69.76	3.566 69.74	3.713 71.78	3.669/3.825 64.94	2.039 21.60
Residue Y	1.685/2.660	3.615	[3.744][a]					
Residue Z						5.114 87.96	3.712/3.421 66.49	
Non-reducing terminus							[3.919/3.698] [64.31]	
Non-reducing terminus						–	–	2.011 21.08

Structure: Residue A →[→9-D-Neu5Acα2→]$_n$ →9-D-Residue Y-α-(2→9)-Residue Z.
[a] Use of parenthesis indicates that the assignment is tentative.

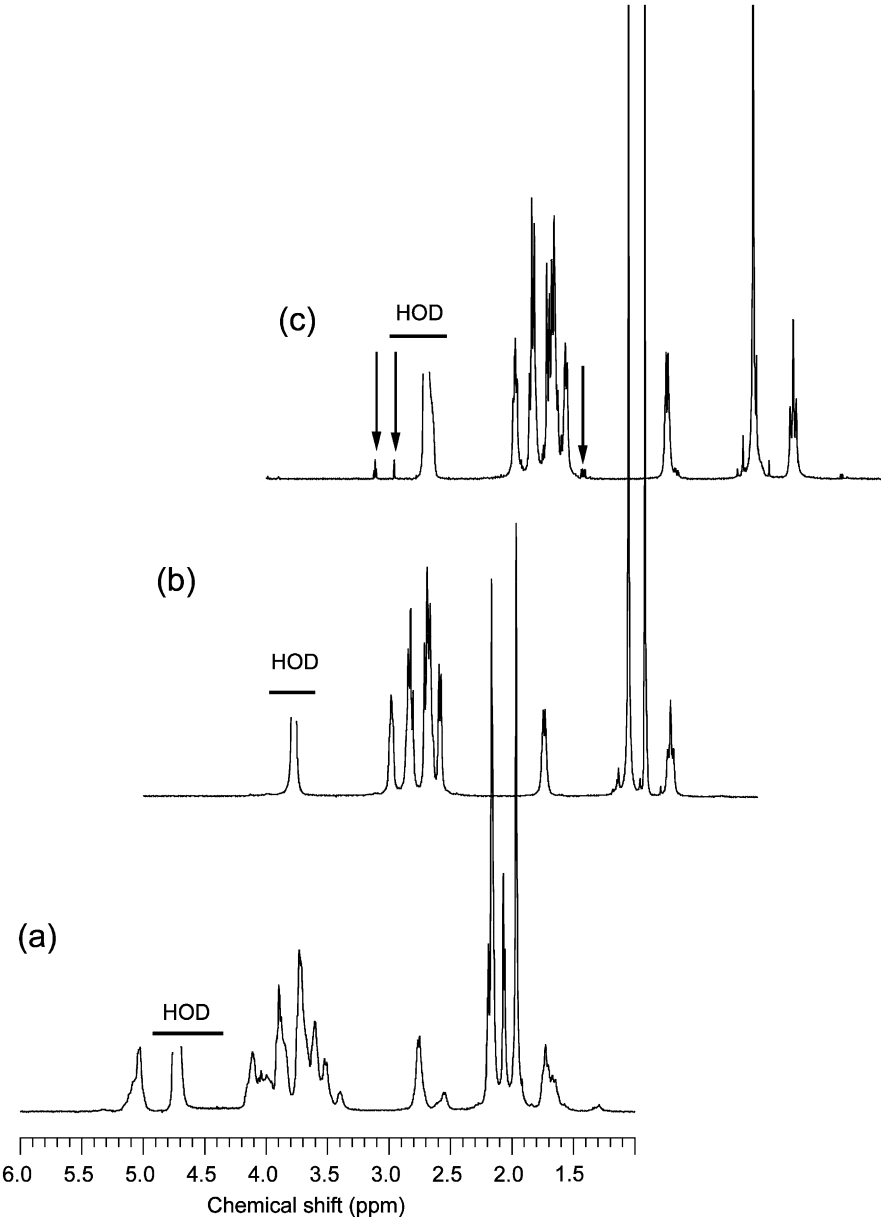

Fig. 5. Overlaid partial 500 MHz NMR spectra of (a) native *O*-acetylated Men C CPS, (b) base de-*O*-acetylated Men C CPS, in 200 mM NaOD, and (c) oligosaccharides derived from periodate oxidation of the de-*O*-acetylated CPS. Resolved resonances arising from end-groups in (c) are indicated with arrows. Spectra were obtained at 30 °C and at 500 MHz, using long relaxation times.

Table 3
Proton and ^{13}C NMR Assignments for the de-*O*-acetylated polysaccharides, at 30 °C in 200 mM NaOD

Polysaccharide	H-1 C-1	H-2 C-2	H-3, H-3′ C-3	H-4 C-4	H-5 C-5	H-6, H-6′ C-6	H-7 C-7	H-8 C-8	H-9, H-9′ C-9	Me
Men A	5.426 96.34	4.447 54.33	4.139 69.74	3.786 67.09	3.991 73.47	4.246, 4.172 65.54				2.078 23.13
De-*O*-Ac Men C			1.711, 2.747 41.16	3.669 69.31	3.826 52.82	3.710 73.12	3.588 69.07	3.980 71.09	3.848, 3.678 66.12	2.052 22.97
De-*O*-Ac Men Y →4Neu5Acα2→ →6Glc*p*α1→	5.024 96.80	3.530 72.08	1.694, 2.872 37.99 3.572 73.94	3.702 75.02 3.498 70.37	4.037 50.75 3.620 71.92	3.782 73.30 4.010, 3.652 63.66	3.640 69.35	3.920 73.02	3.652, 3.878 63.66	2.033
De-*O*-Ac Men W135 →4Neu5Acα2→ →6Gal*p*α1→	5.056 96.26	3.789 68.98	1.667, 2.880 37.81 3.721 70.35	3.695 74.43 3.977 70.27	4.041 50.67 3.841 70.81	3.819 73.34 3.902, 3.659 63.28	3.638 69.41	3.884 73.16	3.675, 3.890 63.94	2.091 23.65

3.6. Chemical shift changes with alterations in sample temperature, sample quantity and NaOD concentration

To test the robustness of the method, 1D ^{1}H spectra were collected on the Groups C, W135 and Y CPSs at neutral pH, and (for all CPSs) in 50, 100, 200 and 400 mM NaOD at 30 °C, and in 200 mM NaOD at 50 °C. To summarised these data, in the Group A CPS, chemical shifts for the H-1 and *N*-acetyl methyl resonances changed negligibly between 50 and 400 mM NaOD, whilst the following changes were observed (upfield unless stated)—H-2: 0.027 ppm; H-3: 0.013 ppm, H-4: 0.037 ppm: H-5: 0.015 ppm; H-6: 0.010 ppm downfield and H-6: 0.020 ppm. Chemical shift differences between spectra collected at 30 and 50 °C were ca. 0.003 ppm except the H-5, which moved by 0.011 ppm. In the Men C CPS, the resolved proton resonances all move upfield, H-3a by 0.025 ppm, H-3e by 0.016 ppm, H-7 by 0.013 ppm and H-8 by 0.008 ppm. The *N*-acetyl methyl resonance is unchanged. Changing the sample temperature caused very small changes (0.012 ppm or less) for all resonances apart from H-4 and H-9' (0.021 ppm and 0.022 downfield, respectively). For both the Groups W135 and Y, the chemical shifts of resolved resonances in spectra of CPS in increasing concentrations of NaOD differ very little, with the largest difference being the Gal H-4 in W135 (0.017 ppm) and the Glc H-1 in Y (0.011 ppm). Clear changes occur in unresolved resonances that affect the shape of the envelope, however. Increasing the sample temperature from 30 to 50 °C causes a number of quite significant changes in chemical shift, notably the Gal H-5 (0.015 ppm downfield), Neu5Ac H-4 (0.024 ppm downfield) in Group W135, and Glc H-6' (0.043 ppm downfield) and the Neu5Ac H-4 (0.028 ppm downfield) in the Group Y CPS. No differences were observed in the chemical shifts of resonances from the Men W135 and Men Y when the sample quantity was increased from 0.5 to 5 mg per 350 μl of deuterated water, in 200 mM NaOD.

3.7. Comparison of the ^{1}H and ^{13}C chemical shifts of the CPSs under neutral conditions or in 200 mM NaOH

One manufacturer produces a Men C conjugate vaccine with de-*O*-acetylated Men C glycans by reductive amination of periodate oxidation-derived oligosaccharides to the carrier protein [35,36]. Control of the identity and purity of the

saccharide component is feasible either at the de-*O*-acetylated CPS or oligosaccharide stage, and at neutral pH. We explored changes in chemical shift between de-*O*-acetylated CPS under neutral conditions or in 200 mM NaOD. As there is growing interest in Men Y and Men W135 conjugate vaccines and available evidence [37] suggests that *O*-acetylation of the CPS may not be important in eliciting protective immunity, we also determined the dependence of the spectra of the de-*O*-acetylated Men Y and Men W135 CPSs under neutral and basic conditions. Comparison of the one-dimensional ^{1}H NMR spectra of the Men W135 and Y CPSs in 200 mM NaOD with samples of the material under neutral pH showed noticeable differences in chemical shifts.

3.8. Stability of the de-*O*-acetylated CPS on extended storage in basic conditions

Two factors are likely to be important here, if there is a delay between base addition and spectral acquisition: base-catalysed de-*N*-acetylation which will create an error in the quantification of the *O*-acetyl content of the original CPS, and base-catalysed degradation of the polysaccharide backbone, which will create spurious resonances which influence the ability to assign the identity of the CPS.

3.8.1. Group A CPS

Sample introduced into the spectrometer within 3 h of base addition and kept at room temperature showed no signs (additional minor resonances) suggesting depolymerisation had occurred, and the spectrum of material stored in 200 mM NaOD at 4 °C for 11 days was largely identical to that of freshly de-*O*-acetylated material. Five minor resonances were observed between 1.75 and 2.25 ppm, with intensities between 0.5 and 1% of that of the *N*-acetyl resonance. The ratio of the integrals of the *N*-acetyl and acetate anion resonances changed slightly after 11 days at 4 °C, corresponded to an increase in the calculated degree of *O*-acetylation of the native CPS from 89.9 to 91.4%, although this figure is sensitive to the phasing of the peaks. The relative intensities of the five minor resonances did not change, and no minor peaks suggestive of non-*N*-acetylated ManNAc were observed. These data indicate that sample may be stored under basic solution at 4 °C for periods in excess of a week without compromising the test result or changing the determined *O*-acetyl content in excess of the estimated error ranges (see below).

3.8.2. Groups C, W135 and Y CPSs

The saccharide backbone of the Men C CPS is stable under the aggressively basic conditions used to de-*O*- and de-*N*-acetylation the CPS prior to re-*N*-acetylation [37], and we found no evidence for saccharide degradation during storage of the Groups C, W135 or Y CPSs in 200 mM NaOD at 30 °C or lower. Maintaining a sample of the W135 CPS at 30 °C for 24 h in 200 mM resulted in no change ($<1\%$, within the expected error limits) in the relative intensities of the *N*-acetyl and acetate anion resonances. Similarly, no differences were observed in the spectra of the Groups Y and W135 when samples in base were stored at room temperature (ca. 21 °C) for 9 days.

3.9. Quantitation of the *O*-acetyl content of the CPS from the NMR spectrum of the native polysaccharide

The degree of *O*-acetylation of the native polysaccharide can be estimated from the relative intensities of key peaks in the spectrum that, due to *O*-acetylation, are well resolved. For the Group A, the simplest approach is to compare the relative integrals of the ManNAc H-1 (several overlapping peaks close to 5.43 ppm) and the cluster at 5.12 ppm, comprising the H-3 of ManNAc(3OAc) residues and the H-4 of ManNAc(4OAc) residues, or by integration of the four resolved ManNAc H-2 resonances [20]. For the Group C CPS, the key resolved resonances from *O*-acetylated residues are the H-7 and H-8 resonances in Neu5Ac(7OAc) and Neu5Ac(8OAc), respectively, with the Neu5Ac H-3e cluster used as a reference [20]. In Groups W135 and Y the Neu5Ac H-7 and H-9

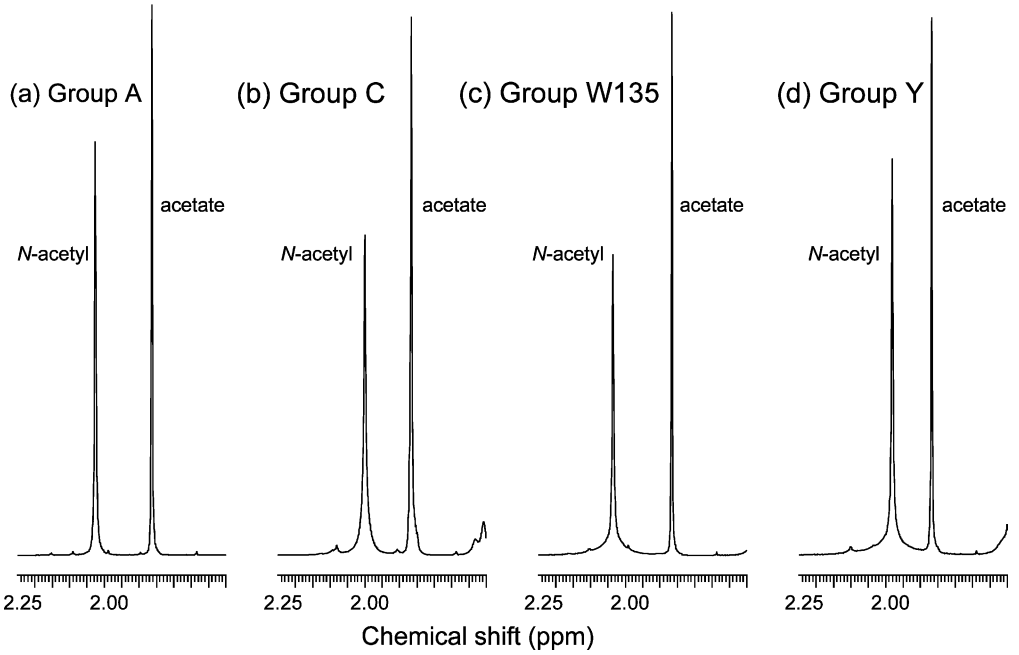

Fig. 6. Partial 500 MHz ^{1}H NMR spectra, obtained at 30 °C, showing the *N*-acetyl and acetate anion regions of de-*O*-acetylated (a) Group A CPS, (b) Group C CPS, (c) Group W135, and (d) Group Y CPS. Whilst in the Groups A and C CPSs the two peaks are well resolved and the acetate content can be readily determined by comparison of the integrals of these resonance, the poor peak shape of the *N*-acetyl resonance in the Groups W135 and Y prevent this.

resonances can be used (the proportion of 8-*O*-acetylation is expected to be very low), with the hexose H-1 acting as a reference peak. The H-7 resonance from Neu5Ac(7OAc) is close to the base of the Hex H-1, complicating integration. These data provide a means to assess the accuracy of the data provided after de-*O*-acetylation.

3.10. Quantitation of the O-acetyl content of the CPS from the integration of the acetate anion resonances and resonances arising from the CPS

The presence of acetate anion in the native CPS sample will obviously introduce errors, or require correction. Comparing about 50 spectra from all serogroups, most samples contained approximately 1–2% of acetate (mol/mol compared to CPS repeat unit), with two samples containing higher levels (6–7.5%). In most cases we have found that the experimental determination of the *O*-acetyl content are so far in excess of the specification that correction for free acetate is unnecessary, but it is obviously possible.

Base-catalysed de-*O*-acetylation releases acetate anion, and comparison of the integral of this resonance with that of an appropriate resonance from the CPS allows the original degree of *O*-acetylation to be determined. Several integrations were performed using different resonances from the CPSs, and in general the use of the Neu5Ac H-3e resonance, close to 2.9 ppm, was preferred, as it has good peak shape, does not overlap with other resonances and baseline correction close to this peak is simple. The simplest option, comparison of the areas of the *N*-acetyl and acetate ion resonances was unsatisfactory in some cases, due to the poor peak shape of the *N*-acetyl resonance (Fig. 6). The data and statistical analyses are summarised in Table 4. Averages and ranges for each sample analysed are reported, from several ap-

Table 4
Comparison of the *O*-acetyl contents of the CPSs determined by different methods

Serogroup	No. of samples	Native CPS			De-*O*-acetyl		Linear fit	Correlation
		[A][a]	[B][a]	[C][a] Acetate vs. NAc	[D][a] Acetate vs. H-1	[E][a] Acetate vs. H-3		
		Average[b] Range[b] CV	Average[b] Range[b] CV	Average[b] Range[b] CV	Average[b] Range[b] CV	Average[b] Range[b] CV	Average Range	vs. 'Native'
Group A	11	83.5% 72.7–90.2% 1.3%	85.4% 76.0–90.2% 1.3%	88.3% 81.3–95.0% 0.5%			[C] = 1.040 [A] +1.87	0.9965 [C]
Group C	15	84.4% 69.0–90.3% 2.5%		89.9% 76.5–95.7% 0.5%		88.2% 74.9–94.0% 0.3%	[E] = 1.020 [A] +2.06	0.996 [E]
Group W135	10	60.6% 53.6–75.0% 0.7%		66.2% 59.8–86.0% 0.8%	64.8% 57.2–85.4% 2.3%	63.0% 57.0–81.0% 0.7%	[E] = 1.029 [A] +2.36	0.996 [E]
Group Y	10	36.4% 16.6–67.7% 38.6%[c] 28.3–67.7%[c] 1.3%		47.2% 22.3–84.5% 49.3%[c] 37.5–84.5%[c] 1.0%	43.6% 20.8–81.0% 46.1%[c] 35.1–81.0%[c] 0.5%	42.4% 19.6–78.6% 45.0%[c] 33.1–78.6% 0.3%	[E] = 1.168 [A] +0.05	0.9995 [E]

[a] [A] Ratio of acetylated H-3 and H-4 to H-1(Men A) or acetylated H7 and H-8 to H-3e (Men C) or acetylated H-7 and H-9 to Neu5Ac H-3e [Men W135 and Men Y], [B] ratio of H-2 resonances in acetylated and non-acetylated residues, [C] ratio of acetate and *N*-acetyl resonances, [D] ratio of acetate and Hex H-1 resonances, and [E] ration of acetate and Neu5Ac H-3e resonances.
[b] Excluding deliberated de-*O*-acetylated samples.
[c] Excluding an old sample which may not be representative of material currently in production.

proaches. Coefficients of variation are derived from five replicates of a single sample, and correlation between data obtained with native and de-*O*-acetylated CPS. The correlation and linear fitting include data obtained on purified de-*O*-acetylated material.

Coefficients of variation are typically 1–2.5% for native CPS and smaller (usually below 1%) with de-*O*-acetylated samples. Determinations using native and de-*O*-acetylated samples correlate at 0.995 or better, and, for three of the four CPSs, linear fitting of data from the two approaches produced a line with gradient close to one, intersecting the *Y*-axis at close to the origin. The exception to this is the Group Y CPS, where the slope of the line is 1.17. This is number is consistently greater that one for all three integration routines used, ranging between 1.17 and 1.28 depending on the reference peak used. This simplest explanation for this would be the presence of an additional *O*-acetylation site(s) in the native CPS with a low degree of occupancy.

These data show that all the samples of material from the manufacturers complied with the WHO and European specifications for these products. A single sample of the Group Y CPS, produced in the 1980s, shows markedly a lower degree of *O*-acetylation close to the specification. It is not clear if this material is representative of material in current use, and a recent sample for the same serotype from the same manufacturer showed a very high degree of *O*-acetylation. Additionally, as there is no direct evidence of the importance of the *O*-acetyl groups in eliciting protective immunity in man, or on the differential immunogenicity of samples with similar degrees of *O*-acetylation, and

it is not clear what the clinical effect of this would be.

Current pharmacopoeial specifications for the degree of *O*-acetylation of polysaccharide-based vaccines have conventionally been in terms of the number of mmoles of *O*-acetyl per gram dry weight of polysaccharide, coupled with other specification that effectively define a minimum purity for the CPS (e.g. % Neu5Ac, or % phosphorus). Two factors have changed in recent years. Firstly, the power of spectroscopic methods to determine detailed structures for polysaccharides, so that it would seem unlikely that a novel polysaccharide-based vaccine could be licensed without a detailed knowledge of its structure. Secondly, the continuing growth in the availability, use and power of spectroscopic methods. We believe that as monographs and WHO Guidelines are revised, it would be advantageous to cite the 'primary' specification in terms of the proportion of repeat units that carry *O*-acetyl groups, as this is a more obviously relevant. Recalculation in traditional terms remains an option.

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