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Use and Validation of an NMR Test for the Identity and *O*-acetyl content of the *Salmonella typhi* Vi Capsular Polysaccharide Vaccine

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Introduction

Typhoid fever remains a major killer, causing approximately 600 000 deaths per year and many millions more people will suffer a non-fatal infection.[1] Multi-drug resistance is an increasingly common problem with *Salmonella typhi*, and effective treatment requires access to expensive third generation cephalosporin and fluoroquinoline antibiotics, purchase of which imposes a huge strain on Third World health budgets.[2] The purified Vi capsular polysaccharide (CPS)[3] from *S. typhi* is currently used as a vaccine and has an efficacy of about 70%.[4–6] Each dose of the vaccine containing 25 μg of CPS.[7] Two key tests in the control of the bulk polysaccharide are an immunological identity test and an estimation of the degree of *O*-acetylation. The *O*-acetyl group has been shown to be a dominant epitope on the polysaccharide[8] and to be immunodominant.[9] Early attempts to produce an effective Vi vaccine failed due to de-*O*-acetylation of the CPS.[10] Current WHO guidelines and the EP monograph for the vaccine bulk prescribe ''a suitable immunochemical method'' to identify the CPS and the Hestrin colorimetric test to assess the degree of *O*-acetylation.[11,12]

CPSs from *Haemophilus influenzae* type b (Hib), *Neisseria meningitidis* and *Streptococcus pneumoniae* have been covalently attached to suitable carrier proteins to improve their efficacy as vaccines. Conjugate vaccines using the Vi CPS are under development.[13–16] Such vaccines are expected to have improved efficacy in young children and this should lead to an increase in immunisation against typhoid.

The Vi antigen is expressed by a number of organisms, including *Citrobacter freundii*,[17] *Salmonella typhi* and *Salmonella paratyphi*,[18] and NMR spectroscopy has been used to demonstrate the structural identity of Vi CPS from these various organisms. The Vi CPS has a monosaccharide repeating unit with the structure →4Gal*p*ANAc(3OAc)*a*→,[17] and therefore the spectrum should contain five resonances from the anomeric or ring hydrogens and two three-proton singlets from the *N*- and *O*-acetyl groups. Additional complexity will arise from incomplete *O*-acetylation of the sample or the presence of impurities. As the native Vi CPS has very high molecular weight, observation of endgroup resonances is not expected. The molecular weight and expression of the Vi CPS are comparable to the *E. coli* Type 1 CPS[3,19] which are linked to a Lipid A moiety (as for lipopolysaccharide) to attach the CPS to the membrane, although no evidence has been presented for the presence or identity of this lipid moiety in the *S. typhi* Vi. The Vi capsule is always co-expressed with the O9 antigen,[3] in which the O-chain of the LPS has a branched tetrasaccharide repeating unit with the unusual 3,6-dideoxy*ribo* sugar tyvelose (Tyv) as a sidechain.[20]

→2D-Man*p*a1→2L-Rha*p*a1→3D-Gal*p*a1→
3
|
a-Tyv*p*

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We have adopted an NMR method to confirm the identity of Vi CPS in bulks to be used in vaccine production and submitted for independent quality assessment at NIBSC. This method differs from similar NMR identity tests for the CPSs from Hib, *N. meningitidis*[21] or *S. pneumoniae* in that the polysaccharide is deliberately degraded prior to spectral analysis, to improve the quality of the spectrum and allow quantitation of the *O*-acetyl content. In this paper we report the test conditions we have developed, our validation of the test and our experience of its use.

Materials and methods

Standard experimental conditions

NMR spectra were acquired on a Varian Unity 500 NMR spectrometer equipped with a 5 mm triple resonance probe (for spectra collected at temperatures above 50°C) or a 5 mm PFG triple resonance probe (for spectra collected at or below 50°C), high precision temperature controller, and under the control of VNMR version 5.3 B. Weighed samples (typically 2–3 mg) of the polysaccharide were dissolved in deuterated water (500 μl; M&G Chemicals, Glossop), lyophilised and redissolved in deuterated water (700 μl) for analysis. De-*O*-acetylated samples were prepared by addition of 40% sodium deuteroxide in deuterated water (MSD Isotope, Montreal, Canada, 15 μl, corresponding to a final concentration of ca. 200 mM) to the sample in the NMR tube. For every control sample, two spectra were 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 sec and a relaxation delay of 25 sec is included, giving a recycle time of 29 sec. Typically 64 scans are collected and averaged (giving a total acquisition time of 30 min). Spectra were Fourier transformed after applying a 1 Hz line broadening function and referenced relative to the acetate anion resonance at 1·91 ppm. The baseline is corrected by the spline method using standard VNMR software procedures, with the zero reset points being 12·3 to 9 ppm, 3·3 to 2·6 ppm and $-0{\cdot}5$ to $-2{\cdot}7$ ppm. 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.

Validation experiments

Spectral assignments for the native and de-*O*-acetylated Vi CPS were obtained by a range of standard homo- and heteronuclear correlation experiments. Spectra were obtained at a nominal probe temperature of 30°C (de-*O*-acetylated polysaccharide one-dimensional spectra and TOCSY spectra), at 50°C (for NOESY of the de-*O*-acetylated material, and for the HMQC spectra of both samples) or 80°C (for the one-dimensional and homonuclear correlation spectra of the native CPS). Standard Varian pulse sequences were used. The mixing time in the TOCSY spectrum was 20 or 35 msec, and 100 or 75 msec in the NOESY, for the de-*O*-acetylated and native CPSs respectively. Samples for ^{13}C NMR spectroscopy were prepared by addition of deuterated water (700 μl) to the polysaccharide (15 mg) in the NMR tube, and dissolution of the sample by sonication. Completion of the de-*O*-acetylation reaction was monitored by NMR spectroscopy after introduction of the sample into the NMR spectrometer, and experiments were carried out with a range of base concentrations and spectra acquired at both 50°C and 30°C.

Results and Discussion

The spectrum of the native polysaccharide

Typically, in our laboratory, NMR identity tests on a polysaccharide bulk are carried out by visual comparison of the one-dimensional proton NMR spectrum of the intact ''test'' polysaccharide sample with that of an archived reference spectra from authentic material, with additional confirmation provided by full assignment of the NMR spectrum to ensure that the spectrum is consistent with the reported structure for that polysaccharide.[21] The NMR spectrum is sensitive to the presence of low molecular weight impurities (such as acetate anion), although the presence of protein or nucleic acid contaminants (in Hib PRP) is less easily defined by NMR spectroscopy (N. Ravenscroft, Chiron Vaccines, personal communication). The only treatment of the polysaccharide prior to spectroscopic analysis is to lyophilise the CPS from deuterated water, to exchange labile hydrogen atoms for deuterium.

The one-dimensional NMR spectrum of the native Vi CPS, obtained at 50°C, is shown in Figure 1(a). Even at elevated temperature, the resonances are broad and the spectrum lacks distinctive features apart from the intense unresolved peak near 2·03 ppm arising from the *N*- and *O*-acetyl resonances. This spectrum was acquired without presaturation of the residual water peak as its chemical

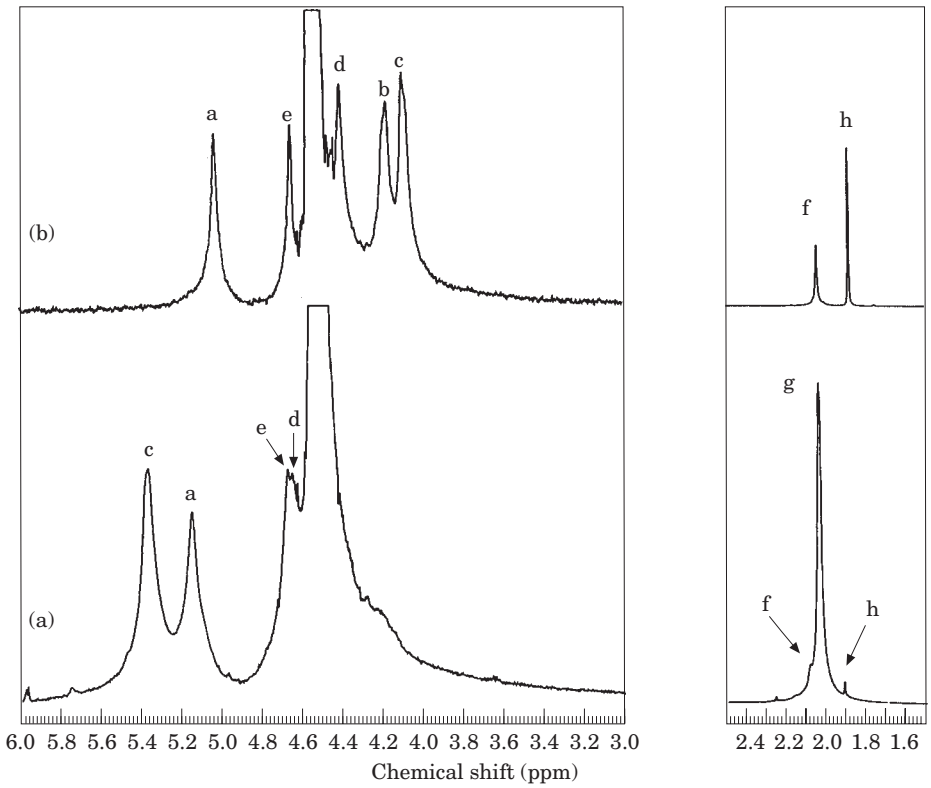

Figure 1. Comparison of the 500 MHz ^{1}H NMR spectra of (a) the native Vi CPS and (b) the same sample after addition of 15 μl of 40% sodium deuteroxide in deuterated water to catalyse de-*O*-acetylation of the polysaccharide, to give a final concentration of base of 200 mM. The spectra were acquired at a sample temperature of 50°C. Peak labelling is as follows: a—H-1; b—H-2; c—H-3; d—H-4; e—H-5; f—*N*-acetyl in non-*O*-acetylated residues; g—*N*-acetyl and *O*-acetyl in *O*-acetylated residues; and h—acetate anion either present in the native Vi CPS or arising during de-*O*-acetylation. Quantitation of the degree of *O*-acetylation is achieved by comparison of the integrals of the resonance from the *N*-acetyl group (at 2·065 ppm) and that from acetate anion (at 1·91 ppm). The vertical scaling factor for the high field region of the spectrum has been reduced by a factor of 35.

shift nearly coincides with that of the H-2 and, due to the slow molecular re-orientation of this polysaccharide, irradiation of the H-2 resonance leads to saturation of the complete spin system through a process of spin diffusion. The proton NMR spectrum of the native CPS was assigned from TOCSY spectra and the ^{13}C spectrum could be assigned from an HMQC spectrum. These assignments are given in Table 1. The spectrum of the Vi CPS has been previously reported,[13] but assignments were not given. The spectra of material from different manufacturers are essentially identical, except when minor contaminants are present (Figure 2).

The spectrum of the de-O-acetylated polysaccharide

Daniels *et al.* commented on the use of base de-*O*-acetylation to reduce the linewidth of resonances in the ^{13}C NMR spectrum of the of Vi polysaccharide.[18] Addition of sodium deuteroxide in deuterated water to the sample causes rapid de-*O*-acetylation and improvement in the proton spectrum, with better spectral dispersion and sharper lines [Figure 1(b)]. Five resolved resonances of approximately equal intensity are observed from the protons of the sugar ring. The linewidth at half height for the H-1 resonance in the native CPS is approximately 30 Hz, and is reduced to approximately 15 Hz in the de-*O*-acetylated material. The spectrum of the de-*O*-acetylated sample is further simplified, as the degree of *O*-acetylation of the native CPS is typically 90%. De-*O*-acetylation does not appear to be directly responsible for the sharpening of the resonances, as the proton NMR spectrum of neutralised de-*O*-acetylated material showed the same broad lines as the native CPS. In our opinion, the spectrum of the de-*O*-acetylated material obtained in alkaline medium is better suited for use as an identification test than that of the native CPS.

Table 1. NMR Assignments for the native and de-*O*-acetylated *S. typhi* Vi CPS

Residue	H-1	H-2 NAc	H-3 OAc	H-4	H-5	
	C-1	C-2 NAc	C-3 OAc	C-4	C-5	C-6
→4GalNAcA(3OAc)α1→	5·13	4·53 2·01	5·34 2·03	4·63	4·66	
	101·41	50·01 24·98/177·16	72·56 22·79/177·16	78·88	74·85	175·97
→4GalNAcAα1→	5·10	4·19 2·065	4·13	4·44	4·70	
	101·3	52·5 25·3/177·7	70·4	80·8	74·1	176·3
Resonances from O9 LPS						
Tyv	4·932	4·074	1·818 2·070	3·641	3·784	1·290
	102·2		34·22			17·58
Rha				3·562	3·880	1·358 17·78

Data obtained at 50°C. Chemical shifts referenced to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) at zero ppm.

The proton and ^{13}C NMR spectra of the de-*O*-acetylated material were assigned (Table 1) using similar approaches to those used for the native CPS. The assignment of the ^{13}C spectrum is fully consistent with that reported previously.[18] In the proton NMR spectrum, well-resolved *N*-acetyl and acetate resonances are observed, so that the degree of *O*-acetylation of the original CPS may be determined by comparison of the integrals of these two resonances. The spectra of different batches of material from the same manufacturer showed a high degree of consistency, and the spectra of material from different manufacturers were essentially identical.

Robustness of the assay to variation in the experimental conditions

De-*O*-acetylation was carried out by addition of 8, 15 or 35 μl of 40% NaOD, corresponding to final base concentrations of approximately 100, 200 and 500 mM. The concentration of the Vi polysaccharide ''repeat unit'', assuming 3 mg of material is used in a 700 μl final volume, is approximately 16 mM: the base is always present in a large molar excess. At the highest base concentration, some precipitation was observed, but the precipitate was not identified. The spectra of the polysaccharide were essentially identical at all three base concentrations, as assessed by the chemical shifts and line widths of the major resonances. An attempt was made to monitor the time course of the de-*O*-acetylation reaction, using NMR spectroscopy. Using a final base concentration of 200 mM and acquiring the spectra at 50°C, de-*O*-acetylation was complete before the sample had equilibrated and the spectrometer shimmed (approximately 15 min after addition of the base to the CPS sample), whilst at 30°C, using 100 mM base, only the second half of the reaction could be observed. This was apparent from the disappearance of the peak at 2·03 ppm, assigned as the coincident *O*- and *N*-acetyl resonances from *O*-acetylated residues, which reduced in intensity with time, and a corresponding increase in the intensity of an *N*-acetyl resonance at 2·065 ppm and the acetate anion resonance at 1·908 ppm. Therefore, under our standard conditions, using a final base concentration of 200 mM and with data collection at 50°C, the spectrum can be collected as soon after the addition of base as is practicable.

At elevated temperature in strong base the polysaccharide degrades. No spectrum could be obtained on a sample of the Vi CPS kept in 500 mM base at 80°C for two days, whilst a sample maintained at 80°C in 200 mM base underwent slow de-*N*-acetylation (approximately 5% over 1 h). As judged by the NMR spectrum, even in 500 mM base the polysaccharide was stable after storage at 4°C for two days. Therefore, spectra are obtained on samples as soon after addition of base as is practicable, and samples are kept at 4°C, if required.

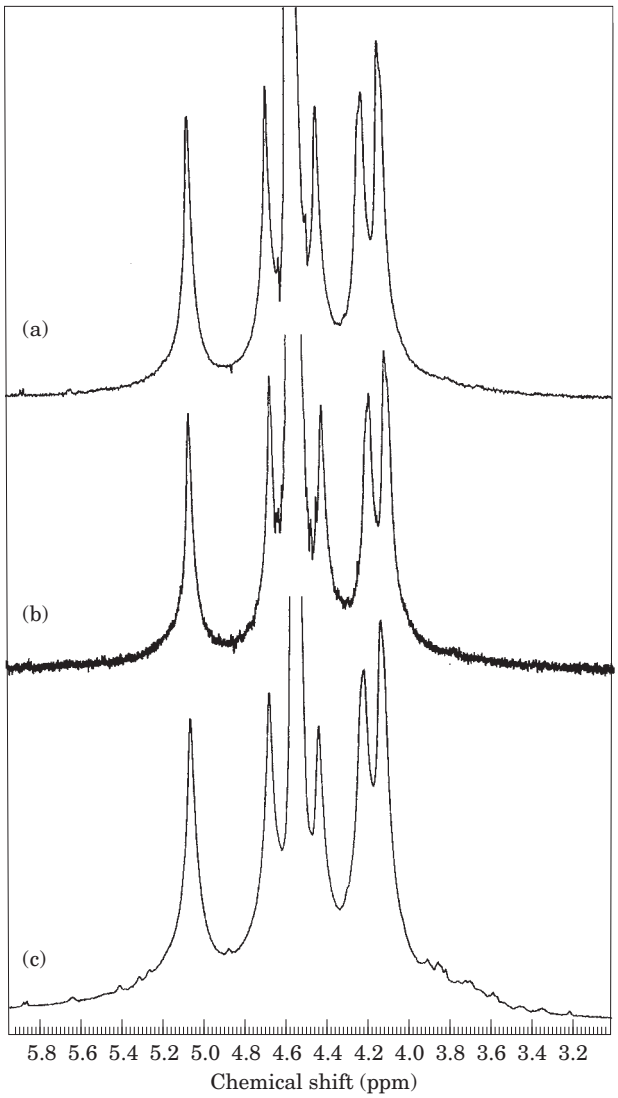

Figure 2. Partial one-dimensional 500 MHz ^{1}H NMR spectra of de-*O*-acetylated Vi polysaccharide from three different manufactures, obtained at 50°C.

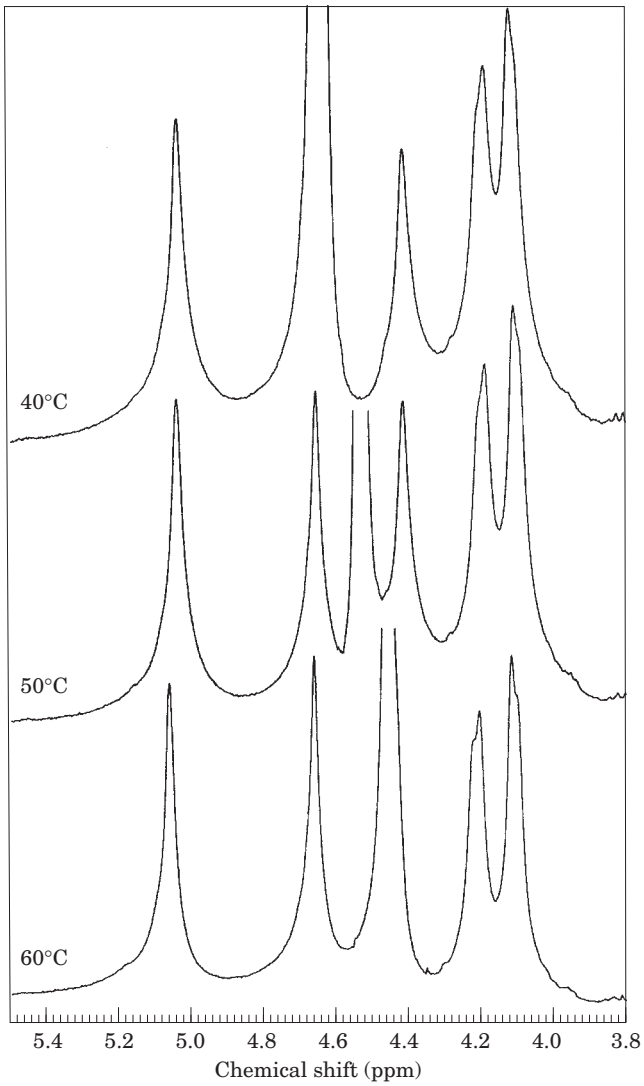

Figure 3. Partial one-dimensional 500 Mz ^{1}H NMR spectra of the de-*O*-acetylated Vi CPS acquired at samples temperatures of (a) 40°C, (b) 50°C and (c) 60°C.

The method is robust against small changes in the temperature at which the NMR spectrum is acquired, with no significant changes in chemical shift for the resonances arising from the CPS when the spectrum is acquired at either 40°C or 60°C (Figure 3). However, at these temperatures, overlap occurs between the resonance from residual water and one of the CPS-derived resonances: the H-5 at 40°C and H-4 at 60°C. The relative integrals of the *N*-acetyl and acetate anion resonances were shown to be independent of the temperature at which the data were collected, between 40°C and 60°C. The position of the resonance from residual water provides an internal control of sample temperature.

For samples received as a solid, deuterium exchange is usually unnecessary, as the samples are sufficiently dry that the resonance from residual water resonance is acceptably small. Samples received in solution are lyophilised and exchanged twice with deuterated water prior to redissolution in deuterated water for spectral analysis.

We have repeated the spectroscopy both of the native and de-*O*-acetylated CPS using amounts of CPS between 0·8 and 3 mg and have observed no differences between the spectra, apart from, obviously, poorer signal-to-noise with smaller samples at specified accumulation times. We have compared the spectra of samples from different manufacturers

and the spectra are virtually identical, apart from some variation in the minor, non-Vi CPS contaminants which are present.

Identification of polysaccharide and non-polysaccharide impurities

A number of minor contaminants have been observed and identified by NMR spectroscopy in samples of the Vi CPS, including formate anion (sharp singlet at δ_H 8·46 ppm), and, in some older samples, a silicone-based antifoam agent (broad resonances near δ_H 2 and zero ppm) which presumably co-purified from the original growth medium. These identifications have been confirmed by obtaining NMR spectra of authentic samples of these impurities. Samples of the native Vi CPS typically also contain traces of acetate anion, which has implications for our NMR method to quantify the *O*-acetyl content of bulk material (see below). Other small molecular weight impurities have been observed, but have not yet been assigned.

The Vi capsular polysaccharide is always co-expressed with the *Salmonella* O9 LPS O-chain,[22] which has a branched tetrasaccharide repeating unit including rhamnose, and the unusual 3,6-dideoxy-sugar tyvelose[20] as a sidechain. Some data on tyvelose model systems have been published,[23,24] although neither an NMR spectrum or the spectral assignments for the O9 LPS repeat have been reported. One experimental sample of Vi CPS, purified from the cell mass, contained a polysaccharide impurity (Figure 4) that two-dimensional NMR analysis suggested was of high molecular weight and contained a tetrasaccharide repeat, including a 3-deoxysugar and Rha. The assignments that could be made (Table 1) were consistent with the identification of this impurity as the *Salmonella* O9 LPS O-chain. Traces of a phenolic compound were also present in this sample.

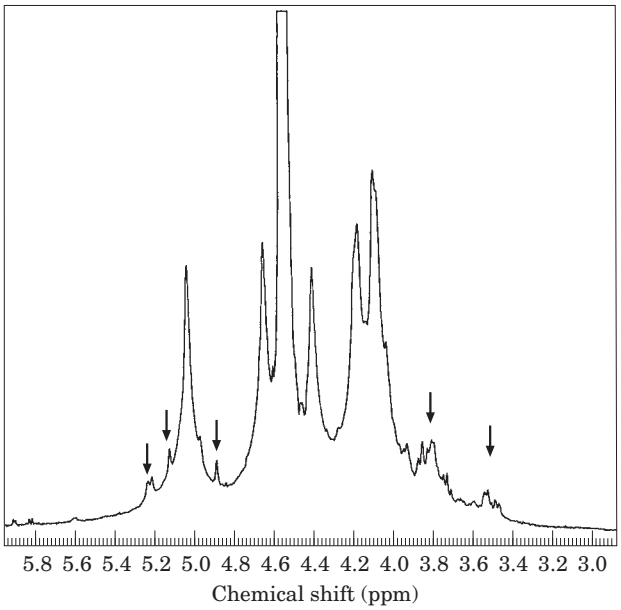

Figure 4. One-dimensional 500 MHz proton NMR spectrum of a sample of de-*O*-acetylated Vi CPS contaminated with the O9 LPS antigen, obtained at 50°C. Resonances arising from the LPS are indicated with arrows.

Quantitation of the O-acetyl content of the polysaccharide

A minimum degree of O-acetylation is specified for Vi vaccines as the immunogenicity is strongly dependent on this parameter. The European Pharmacopoeia specifies ''not less than 2 mmol of ''*O*-acetyl'' per gram of dried polysaccharide'' for bulk polysaccharide, and ''0·085 ($\pm$ 25%) 'μ mole' of *O*-acetyl groups per vaccine dose'' [25 ($\pm$ 20%) μg of polysaccharide].[12] These correspond to $\geq$51·5% of the bulk, assuming the polysaccharide is pure, dry and present as the calcium salt. For the final dosage form, at the limits of both specifications, between 54% and (a physically impossible) 159% of the sugar residues carry *O*-acetyl groups. A direct measure of the proportion of substituted residues would be a more appropriate specification, but unfortunately the Vi content cannot be measured by conventional colorimetric methods and more sophisticated physicochemical[25] or imprecise quantitative immunological approaches are required.[12] The use of NMR for quantitation of the relative amounts of two materials is well known, as long as a there is a sufficient delay between scans to allow full relaxation of the different spins systems being quantified.[26] Comparison of the integrals of the resolved *N*-acetyl and acetate anion resonances in the spectrum of the freshly de-*O*-acetylated material provides an estimate of the *O*-acetyl content of the native CPS, if appropriate acquisition conditions are used. The requirement is to allow a relaxation delay between scans of at least five times the T_1 of the most slowly-relaxing relevant resonance, and, in the light of the slow relaxation of the acetate anion, the relaxation delay needs to be 30 seconds. In this case, the simplest measure is the ratio of the integrals of the *N*-acetyl and acetate anion resonances. The method will always produce a slight overestimate of the *O*-acetyl content of the original polysaccharide, because a trace of acetate anion is present in the original preparation: we estimate

that this is typically 5 mole %, although one manufacturer produces material which contains consistently higher levels of acetate anion (typically 10–15 mole %). It is possible to determine the amount of free acetate anion present in the CPS from the NMR spectrum of the native CPS, and to use this figure to correct the final estimate of the degree of *O*-acetylation of the CPS. Importantly, the estimate of the degree of *O*-acetylation by NMR spectroscopy is a direct measure of the proportion of sugar residues in the CPS which were *O*-acetylated, and does not carry assumptions about the purity of the material, how much water is present, and the counter-ion.

In practice, we have found that the degree of *O*-acetylation is close to 100% for all samples analysed. A shoulder on the lowfield side of the major resonance at 2·03 ppm in the spectrum of the native polysaccharide [Figure 1(a), peak f] arises from non-*O*-acetylated residues, and provides a quick, qualitative indication that *O*-acetylation is not quite complete, but standard NMR integration software has not been developed for the accurate integration of unresolved peaks.

For comparison, NMR determination of the degree of *O*-acetylation of three typical batches of the Vi CPS indicated that 106, 105 and 104% of the residues were *O*-acetylated, whilst the values obtained using the Hestrin methodology were 91, 87 and 85% respectively (recalculated from data provided by the manufacturer). To test reproducibility, a single sample was divided into three equal parts, which were analysed separately. No differences were observed between the spectra of the three materials, and the calculated degrees of *O*-acetylation were the same. For one sample of material, the ratio of the integrals of the *N*-acetyl and acetate anion peaks were the same independent of the temperature at which the spectrum was obtained, between 40°C and 60°C.

The need to leave long relaxation times between scans limits the rate at which spectral data can be accumulated, and with the spectrometer at our disposal we request at least 2 mg of polysaccharide. With less material we need to increase spectral accumulation times: with 1 mg of material the accumulation needs to be four times longer to achieve the same signal-to-noise ratio.

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References

1. Ivanoff B, Levine MM, Lambert PH. Vaccination against typhoid fever: present status. Bull WHO 1994; 72: 957–971.
2. Mourad AS, Metwally M, Deen ANE. Multidrug-resistant *Salmonella typhi*. Clin Infect Dis 1993; 17: 135–136.
3. Virlogeux-Payant I, Popoff MY. The Vi antigen of *Salmonella typhi*. Bull Inst Pasteur 1996; 94: 237–250.
4. Tacket CO, Ferreccio C, Robbins JB *et al*. Safety and immunogenicity of two *Salmonella typhi* Vi capsular polysaccharide vaccines, J Infect Dis 1986; 154: 342–245.
5. Acharya IL, Lowe CU, Thapa R *et al*. Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of *Salmonella typhi*. A preliminary report. N Eng J Med 1987; 317: 1101–1104.
6. Klugman KP, Gilbertson IT, Koornhof HJ *et al*. Protective activity of Vi capsular polysaccharide vaccine against typhoid fever, Lancet 1987; 2 (8569): 1165–1169.
7. Klugman KP, Koornhof HJ, Robbins JB, Le Cam NN. Immunogenicity, efficacy and serological correlates of protection of *Salmonella typhi* Vi capsular polysaccharide vaccine three years after immunization. Vaccine 1996; 14: 435–438.
8. Szewczyk B, Taylor A. Immunochemical properties of the Vi antigen from *Salmonella typhi* Ty2: presence of two antigenic determinants. Infect Immun 1980; 29: 539–544.
9. Szu SC, Li XR, Stone AL, Robbins JB. Relation between structure and immunogenicity of the Vi capsular polysaccharide. Infect Immun 1991; 59: 4555–4561.
10. Robbins JB, Robbins JD. Re-examination of the protective role of the capsular polysaccharide (Vi antigen). J Infect Dis 1984; 150: 436–449.
11. British Pharmacopoeia 1998 pages 2086–7. European Pharmacopoiea 3rd Edition, 1997: 1683–1685.
12. WHO Technical Report. 1994; 840: 14–29.
13. Szu SC, Stone AL, Robbins JD *et al*. Vi capsular polysaccharide-protein conjugates for prevention of typhoid fever. Preparation, characterisation and immunogenicity in laboratory animals. J Exp Med 1987; 166: 15101–524.
14. Szu SC, Li XR, Schneerson R *et al*. Comparative immunogenicities of Vi polysaccharide-protein conjugates composed of cholera toxin or its B subunit as a carrier bound to high- or lower-molecular-weight Vi. Infect Immun 1989; 57: 3823–2827.
15. Szu SC, Taylor DN, Trofa AC *et al*. Laboratory and preliminary clinical characterisation of Vi capsular polysaccharide-protein conjugate vaccines. Infect Immunity 1994; 62: 4440–4444.
16. Kossaczka Z, Bystricky S, Bryla DA *et al*. Synthesis and immunological properties of Vi and di-*O*-acetyl

pectin protein conjugates with adipic acid dihydrazide as the linker. Infect Immun 1997; 65: 2088–2093.
17. Heyns K, Kiessling G. Strufturaufklarung des Vi-Antigens aus *Citrobacter freundii (E. coli)* 5396/38. Carbohydr Res 1967; 3: 340–353.
18. Daniels EM, Schneerson R, Egan WM *et al*. Characterisation of the *Salmonella paratyphi* C Vi polysaccharide. Infect Immun 1989; 57: 3159–3164.
19. Jann K, Jann B. Structure and biosynthesis of the capsular antigens of *Escherichia coli*, in Current Topics in Microbiology and Immunology, Vol. 150 'Bacterial Capsules'. (Ed. Jann K, Jann B), Springer Verlag, Berlin, 1990.
20. Hellerquist CG, Lindberg B, Svensson S *et al*. Structural studies on the O-specific side chains of the cell wall lipopolysaccharides from *Salmonella typhi* and *S. enteriditis*. Acta Chem Scand 1969; 23: 1588–1596.
21. Lemercinier X, Jones C. Full ^{1}H NMR assignments and detailed *O*-acetylation patterns of capsular polysaccharides from *Neisseria meningitidis* used in vaccine production. Carbohydr Res 1996; 296: 83–96.
22. Nolan CM, LaBorde EA, Howell RT, Robbins JB. Identification of *Salmonella typhi* in faecal specimens by an antiserum-agar method. J Med Microbiol 1980; 13: 373–377.
23. Iversen T, Bundle DR. Antigenic determinants of of *Salmonella* serogroups A and D_1. Synthesis of trisaccharides glycosides for use as artificial antigens. Carbohydr Res 1982; 103: 29–40.
24. Probert MA, Zhang J, Bundle DR. Synthesis of α-and β-linked tyvelose epitopes of the *Trichinella spiralis* glycan: 2-acetamido-2-deoxy-3-O-(3,6-dideoxy-D-arabino-hexopyranosyl)-β- D-galactopyranosides. Carbohydr Res 1996; 296: 149–170.
25. Stone AL, Szu SC. Application of optical properties of the Vi capsular polysaccharide for quantitation of the Vi antigen in vaccines for typhhoid fever. J Clin Microbiol 1988; 26: 719–725.
26. Harris RK. Nuclear Magnetic Resonance Spectroscopy: a Physicochemical View, Longmans, Harlow, 1986, page 112.

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