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▲⟨198⟩ NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY IDENTITY TESTING OF BACTERIAL POLYSACCHARIDES USED IN VACCINE MANUFACTURE

1. INTRODUCTION AND SCOPE

This chapter describes the application of nuclear magnetic resonance (NMR) spectroscopy to the identity testing of bacterial polysaccharides used in vaccine manufacture. The identity of the saccharide component in polysaccharide and glycoconjugate vaccines should be confirmed at various stages of the manufacturing process, including bulk monovalent polysaccharide, blended polysaccharide bulk, activated polysaccharide (if isolated), bulk monovalent conjugate, blended conjugate bulks, and final fills. NMR is an appropriate method to confirm the identity of polysaccharides, although it is most useful for bulk monovalent polysaccharides and activated polysaccharides (if isolated). This chapter describes the use of NMR spectroscopy for this purpose, but alternative validated approaches may also be suitable.

For additional information on NMR, including its general principles and applications, see *Applications of Nuclear Magnetic Resonance Spectroscopy* ⟨1761⟩. More specific information on instrument qualification, procedure validation, and reference standards are included in *Nuclear Magnetic Resonance Spectroscopy* ⟨761⟩.

1.1 NMR Spectra

NMR spectra for identity testing are typically collected for samples dissolved in deuterated water (D_2O) or a combination of deuterated water and small amounts of one or more internal standards for chemical shift calibration, quantification, or other purposes. The chemical shift of the resonance from residual water in deuterated solvent (HOD) is more temperature sensitive than other solvent resonances. The spectral separation and resolution of the HOD resonance may be optimized by the choice of temperature at which the spectrum is obtained.

1.2 *O*-Acetylated Polysaccharides

For those polysaccharides that are *O*-acetylated, the consistency of the degree of *O*-acetylation is considered part of the identity test. This could be evaluated through the use of the spectrum of a reference polysaccharide with an acceptable degree of *O*-acetylation, quantification of resonances characteristic of the *O*-acetylation in the spectrum of the intact polysaccharide, or chemical de-*O*-acetylation and relative quantification of the resulting acetate anion compared to the polysaccharide (see *2.4 Experimental Procedures*). The implementation of any given approach is likely to be product specific.

For some polysaccharides, a quantitative specification for the minimum degree of *O*-acetylation may exist; the approaches described in this chapter, appropriately validated, can be used to perform that assay. Requirements for the *O*-acetyl content of polysaccharides are typically quoted as a minimum quantity of "*O*-acetyl residues" (measured by a Hestrin assay) in millimoles per gram of dry weight of polysaccharide. This NMR assay method described in this chapter reports a value based on the average number of *O*-acetyl groups per repeat unit. A method to calibrate between the two approaches should be developed during method validation.

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2. PROCEDURE

2.1 Equipment Requirements

A description of a typical NMR spectrometer is given in ⟨1761⟩, and approaches to installation, performance, and operational qualification are given in ⟨761⟩. This section, and the rest of the chapter, refers to specific requirements for the identity testing of bacterial polysaccharides.

NMR SPECTROMETER

An NMR spectrometer with a minimal nominal field strength corresponding to a proton resonance frequency of NLT 400 MHz and with control of the sample temperature should be used. Temperature calibration within ±3° of the desired temperature is sufficient, provided a consistent sample temperature between the test and reference samples is achieved (or demonstrated in method validation), with temperature stability within a run of NMT 0.5°. NMR probes optimized for proton detection and suitable for 5-mm (outside diameter) NMR tubes should be used. Other probe options are possible but should be proven suitable before use.

PROCESSING PARAMETERS

A weighting function may be applied to the free induction decay (FID) before Fourier transform: a suitable line broadening function may be applied, and in some cases 0.3 Hz has been found suitable. After transform of the FID, the phase is adjusted to pure ▲absorption▲ (ERR 1-Jul-2018) phase. Baseline correction should be applied consistently.

NMR TUBES

These should be of a quality suitable for use in high-field spectrometers and qualified for use in the assay.

2.2 Reagents for Vaccine Polysaccharide Sample Solutions

SOLVENTS

Deuterated solvents subject to proton exchange with the polysaccharide [e.g., deuterated water (D_2O)] must be selected with the highest deuterium proportion (e.g., >99.9 atom % D).

CHEMICAL SHIFT REFERENCE COMPOUNDS

Sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; preferred), sodium trimethylsilylpropionate (TSP), or a deuterated analogue (TSP–d_4) are classically used and set to 0 ppm for the methyl signals. The reference material is usually added at low amounts [0.1%–0.01% (w/v) has been found to be appropriate] to the deuterated water used to dissolve the final sample.

[NOTE—Deuterated water >99.96% ^2H containing 0.01% DSS (w/v) is commercially available.]

SODIUM DEUTEROXIDE

Forty percent w/v (10 M) sodium deuteroxide (NaOD) in deuterated water (>99 atom % D) is commonly used to induce the de-*O*-acetylation of polysaccharides.

2.3 Sample Requirements

An aliquot of bulk purified polysaccharide or appropriate process intermediates in solid or liquid state containing 0.5–20 mg of saccharide content is suitable for preparation of NMR analytical samples. To obtain a solid aliquot, the relevant amount of material in liquid solution is dried under vacuum (using a freeze-drier, lyophilizer, or other solvent evaporator).

REFERENCE SPECTRA FOR ANTICIPATED IMPURITIES

Reference spectra for anticipated process-related impurities (e.g., ethanol, antifoaming agents, phenol, cetyl trimethylammonium bromide) should be obtained under the same experimental conditions as those used to analyze the test sample and at concentrations found similar to those in routine test samples. These spectra provide evidence for those resonances that may be excluded from further consideration when assigning the identity of the samples.

REFERENCE SPECTRA FOR POLYSACCHARIDES

Reference spectra for the polysaccharides samples being tested should be obtained by collecting spectra of authentic samples under identical sample preparation and spectrometer acquisition and data processing conditions to those used for test samples. Comparison of the spectra of test samples with these reference spectra allows the identity of the test samples to be confirmed.

2.4 Experimental Procedures

Two experimental protocols are described below. The first protocol is suitable for samples in which a single one-dimensional ^1H NMR spectrum will produce all of the required information. The second protocol includes in situ base-catalyzed de-*O*-acetylation of the polysaccharide to release *O*-acetyl groups as acetate anions, both simplifying the spectrum of the polysaccharide and allowing quantification of the degree of *O*-acetylation. A second one-dimensional ^1H NMR spectrum is collected after the de-*O*-acetylation step. The choice of which protocol to adopt will depend upon the product being tested and the information required; many stages in these two protocols are similar (e.g., sample preparation).

SAMPLE PREPARATION

NMR analytical samples of polysaccharide and its derivatives can be prepared as either solid- or liquid-state aliquots. NMR analytical samples are usually prepared by dissolving the solid aliquot in about 0.7 mL of deuterated solvent or, more rarely, by adding at least 10% (v/v) of deuterated solvent to the liquid aliquot. The solution is mixed to obtain a uniform concentration and is subsequently transferred to a 5-mm NMR tube.

[NOTE—NMR instrument design may allow or require the use of smaller volumes; users should confirm this for their instrument configuration.]

DEUTERIUM EXCHANGE

With suitable method validation, lyophilization from deuterium oxide ("deuterium exchange") may be used to reduce the intensity of the resonance from residual water (H_2O)/HOD.

pH

Under some circumstances, control of the pH of the sample may be appropriate.

2.5 Procedure 1a

This procedure uses visual comparison with a reference spectra and/or comparison of test chemical shifts with reference chemical shift values.

SCOPE

This approach is compatible with polysaccharides that lack *O*-acetylation, such as *Haemophilus influenzae* type b or many pneumococcal polysaccharides, or with *O*-acetylated polysaccharides, such as *Neisseria meningitidis* or many pneumococcal polysaccharides where the product and spectral consistency allow identity to be established through direct comparison of test and reference spectra.

NMR SPECTRUM OF THE TEST SAMPLE

The NMR spectrum of the test sample is acquired under the same instrument operational and data processing parameters to those used to obtain the reference spectrum. Confirmation of identity can be based on visual comparison of the test and product-specific reference spectra, comparing resonance position, line width, relative intensity, and multiplicity. The complete spectrum, distinctive regions of the spectrum, or a series of at least five resonances (or three distinctive resonances in polysaccharides with monosaccharide or disaccharide repeat units) can be used in this comparison (as long as these are shown to be diagnostic).

RESONANCES

In a well-understood assay, resonances assigned as deriving from process-related impurities that are known to be removed at a later stage in the manufacturing process or otherwise proven not to impact product quality can be excluded from determination of identity.

2.6 Procedure 1b

This procedure uses analytical samples in deuterated water (D_2O) and a comparison with a reference spectrum by calculation of a correlation coefficient.

SCOPE

This approach is compatible with *Streptococcus pneumoniae* polysaccharides where the consistency of the product and the spectrum allow identity to be established through direct mathematical comparison of test and reference spectra.

With mathematical comparisons, a distinctive region of each spectrum (e.g., 4.64–5.89 ppm) is compared to spectra generated for designated reference samples for each polysaccharide of interest. The similarity of two spectral profiles for a test and reference sample acquired under identical conditions and in the same matrix is evaluated using a correlation coefficient (ρ). A positive identification of the sample polysaccharide is achieved when ρ is NLT 0.95 (or another validated value) between the sample and reference spectra.

2.7 Procedure 2

The procedure is in-tube base-catalyzed de-*O*-acetylation and quantification of the acetate content.

SCOPE

In some cases, the degree of *O*-acetylation and their position, accompanied by spontaneous *O*-acetyl migration in solution, can complicate the NMR profile. Base-catalyzed de-*O*-acetylation of the polysaccharide in the NMR tube has proven useful for the *Salmonella typhi* Vi and the meningococcal Groups A, C, W135, and Y polysaccharides. Adding a base may also reduce line widths.

SAMPLE HANDLING

The sample is prepared as in *Sample Preparation* in *2.4 Experimental Procedures* and a one-dimensional ^1H NMR spectrum is acquired as in *2.5 Procedure 1a*. This spectrum provides information on the presence/amount of acetate anion in the native sample. Sodium deuteroxide (NaOD) in deuterated water (D_2O) is added to the sample in the NMR tube to a final concentration of approximately 200 mM [equivalent to adding 14 µL of 10 M sodium deuteroxide (NaOD) in deuterated water (D_2O) to a

0.7-mL sample]. De-*O*-acetylation is very rapid for almost all polysaccharides, but appropriate conditions should be evaluated in method development and validation. The NMR spectrum of the sample is reacquired after de-*O*-acetylation using acquisition conditions appropriate for quantitative spectroscopy. The spectrum of the de-*O*-acetylated material is characteristic of the polysaccharide backbone, and the degree of *O*-acetylation in the original sample is calculated from the integrals of the acetate anion and an appropriate resonance arising from the saccharide backbone. If the amount of acetate anion in the native sample is low, for example <5%, correction of the intensity of the acetate anion resonance in the de-*O*-acetylated sample is not necessary.

2.8 Assay Criteria

TEMPERATURE

The temperature of the sample should be the same, within limits established during method validation, as that of the reference standard, and the sample temperature can be determined by the chemical shift difference between the HOD resonance and the internal chemical shift reference standard. For example, when using TSP-d_4 as the reference standard, calculate the actual temperature using the following equation based on the chemical shift of the HOD signal:

$$\delta = 5.051 - 0.0111(T)$$

where δ is the chemical shift of the HOD peak, and T is the temperature in Celsius (°).

Other equations apply when different chemical shift reference standards are used. Consistency in the chemical shift difference between the HOD and chemical shift reference standard is a surrogate for sample temperature.

CHEMICAL SHIFT REFERENCE STANDARD

The full width half-height (*fwhh*) line width of a defined resonance from a small molecule (e.g., the chemical shift reference standard) in the test sample should be within limits set during method validation to indicate both acceptable shimming of the magnet and sample temperature stability during the analysis. The signal-to-noise ratio of a defined signal within the spectrum of the polysaccharide material should exceed the specification established during method validation.

2.9 System Suitability

SYSTEM SUITABILITY SOLUTION

Dissolve 1 vial of USP PS NMR System Suitability RS in deuterated water (>99.9% D, 1 mL) containing 0.01% DSS with brief mixing.

[NOTE—For example, for 10 s using a rotary mixer.]

Transfer 0.7 mL of this solution into an NMR tube (5-mm outside diameter).

[NOTE—NMR instrument design may allow or require the use of smaller volumes; users should confirm this for their instrument configuration.]

SYSTEM SUITABILITY: PROCEDURE

Using an NMR spectrometer operating at NLT 400 MHz nominal frequency for ^{1}H, acquire an FID using NLT 64 scans with a 90° pulse, a spectral width of 12 ppm, a recycle delay of NLT 30 s, a number of data points NLT 64,000, and with the spectral window centered at 4 ppm. Record the ^{1}H NMR spectra of the *System suitability solution* at a stable temperature of 25 ± 3°. Obtain the spectrum using the above parameters. Transform the data after exponential multiplication (0.3 Hz line broadening). The DSS methyl signal should be set to 0.00 ppm.

ACCEPTANCE CRITERIA

- The chemical shifts for the *N*-acetyl, GlcA H-2, and GlcNAc H-1 anomeric resonances in the *System suitability solution* should be observed at 2.01 ± 0.05, 3.33 ± 0.05, and 5.15 ± 0.05 ppm, respectively.
- The line width (*fwhh*) of the *N*-acetyl resonance at 2.01 ppm should be NMT 3.5 Hz.

2.10 USP Reference Standards ⟨11⟩

USP PS NMR System Suitability RS

▲ 1S (USP41)