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Applications of NMR Spectroscopy in Biopharmaceutical Product Development

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ABBREVIATIONS

CPMG	Carr–Purcell–Meiboom–Gill
CE	Capillary electrophoresis
HPLC	High-performance liquid chromatography
HEPES	*N*-2-Hydroxyethylpiperazine-*N*″-2-ethanesulfonic acid
LC	Liquid chromatography
MES	2-(*N*-Morpholino)-ethanesulfonic acid
MOPS	3-(*N*-Morpholino)-propanesulfonic acid
^{1}H-NMR	Proton nuclear magnetic resonance
ppm	Chemical shift unit (part per million, Hz/MHz)
TEA	Tetraethylammonium
TFA	Trifluoracetic acid
TMA	Tetramethylammonium
Tris	2-Amino-2hydroxymethyl-1,3-propanediol
UF/DF	Ultrafiltration/diafiltration

NMR AND BIOPHARMACEUTICAL PRODUCT DEVELOPMENT

Ever since its discovery more than 50 years ago, nuclear magnetic resonance (NMR) spectroscopy has been an important analytical method for chemists. The introduction of FT (Fourier transform) NMR techniques in the early 1970s made it even more powerful and widely used. NMR is undoubtedly one of the most valuable tools in the discovery stage of pharmaceutical product development. For example, structural biologists often use NMR to determine the three-dimensional solution structures of biomolecules of pharmaceutical importance.[1–3] In recent years, NMR has also been employed to screen small-molecule combinatorial libraries in search of drug leads that bind to target proteins.[4–6] For a small-molecule drug candidate, NMR is an indispensable analytical technique in the development stage as well. Using NMR to identify drug metabolites, to determine degradation products of a drug, and to elucidate impurities in a drug substance are some important applications of NMR in a small-molecule drug product development program.

In contrast, NMR is fairly underutilized beyond the discovery phase of the biopharmaceutical product development, mainly because the sensitivity and resolution of NMR for macromolecules such as proteins are much lower than those for small molecules. The characterization of a protein product and its variants is a critical part of a protein-based pharmaceutical product development program. The determination of a protein structure using NMR, while providing extremely valuable information, is not trivial and not always feasible. Thus, NMR has not been used often in biopharmaceutical product characterization studies. However, there are still many analytical issues that can readily be addressed by NMR in a biopharmaceutical product development program. For example, process validation studies, as required by the regulatory agencies, must be conducted to demonstrate that impurities (often small molecules) from the cell culture fluids and process buffers used in the manufacturing process are removed from the final product. In addition, the manufacturing process must not cause objectionable levels of extractables from process equipment (filters, columns, gaskets, etc.).[7] Many of these process-related impurities can be easily detected by fairly simple and general NMR methods.

Traditionally, HPLC, GC-MS, or LC-MS methods were used to monitor the clearance of small-molecule impurities. These analytical techniques often require unique solvents, columns, methods, reagents, detectors, and buffers for each analyte to be quantified. The NMR method, albeit not the most sensitive technique, normally does not have these problems. In this chapter, some examples will be used to demonstrate that NMR is a fast, generic, and reliable analytical technique for solving analytical problems encountered in the development of biopharmaceutical products. The NMR techniques described here require minimal sample handling and use simple standard NMR methods. They can easily be implemented and used for process development and validation purposes.

NMR is generally considered an expensive and insensitive technique. Despite higher cost and lower sensitivity, the advantages that NMR offers are significant and may outweigh its weaknesses. In fact, the cost of NMR can be

offset easily by the savings in time and effort to solve biopharmaceutical analytical problems. If the instrument capital cost is a concern, performing these NMR tests in a contract lab might be an alternative. The sensitivity of NMR, while lower than those of other techniques such as mass spectrometry, is often adequate for solving analytical problems in process development and validation.

NMR SPECTROSCOPY

The underlying physical principles of NMR have been established and are well understood.[8] Applications of both solid- and solution-state NMR spectroscopy can be found in many different disciplines. It is routinely used in structural elucidation of organic and inorganic compounds, polymers, and biomolecules (e.g., proteins, nucleic acids, and carbohydrates). Additionally, NMR can be used to study molecular interactions (e.g., protein–protein and protein–ligand), molecular dynamics, and chemical reactions. It has also been used extensively in medical research and imaging (magnetic resonance imaging).

NMR is basically one form of absorption spectroscopy. Interested readers can find details of NMR theory and methodology elsewhere.[8–10] Briefly, NMR signals arise from the transitions between nuclear spin states. In the presence of an external magnetic field, nuclei with nonzero spin angular momentum (for example, a proton has a spin of 1/2) will have nondegenerate nuclear spin states. An electromagnetic radiation applied to a nucleus at a frequency corresponding to the energy difference between these spin states will result in a transition between these spin states. The radiation that induces such transitions is in the radio frequency (RF) range and delicately dependent on the chemical environment of each nucleus in a molecule. A plot of absorption peak intensities vs. frequencies constitutes an NMR spectrum. All modern NMR instruments are operated in FT mode, which uses RF pulses to excite all nuclei in a sample simultaneously and then detects the nuclear free induction decay (FID). During the FID, the excited nuclei return to their equilibrium states while releasing the RF energy, which can be detected by a tuned RF coil. The FT of FID gives rise to a normal absorption spectrum. The FT method allows for much faster multiple-transient acquisition and, therefore, results in much higher sensitivities than the conventional continuous wave (CW) mode of operation.

COMMONLY USED NMR METHODS

There are a large number of one- and multidimensional NMR methods available for solving different analytical problems. Typically, only a few standard NMR experiments are necessary for solving most process-related analytical problems (Table 12.1). These methods are all straightforward and easy to execute routinely. A repertoire of more complex methods can be found elsewhere.[9]

A solvent-suppression method is normally required in NMR applications because the solvent signals, if not suppressed, may saturate the receiver and hinder

Table 12.1 Commonly Used FT-NMR Methods

Experiment	Description	Applications in Process Development and Validation
Standard one-dimensional NMR	One-dimensional NMR — simple one-pulse experiment, typically with presaturation of solvent during the recycle delay with a weak RF field	To quantify small molecules To identify some simple small molecules
CPMG	Carr–Purcell–Meiboom–Gil l spin-echo method	To suppress signals with short relaxation time (e.g., protein signals) Small molecules can be detected and quantified in the presence of proteins
TOCSY	Two-dimensional total correlation spectroscopy	To elucidate structure of organic molecules To establish proton coupling network and molecular connectivity
COSY	Homonuclear correlation spectroscopy	To elucidate structure of organic molecules To establish proton coupling network within a molecule
NOESY	Homonuclear NOE (nuclear Overhauser) spectroscopy	To elucidate structure of organic molecules To determine the spatial proximity of nuclei
HSQC HMQC	Heteronuclear single-quantum/multiple-quantum correlation spectroscopy	To elucidate structure of organic molecules To determine heteronuclear coupling connectivity
HMBC	Heteronuclear multiple-bond correlation spectroscopy	To elucidate structure of organic molecules To establish long-range (i.e., multibond) heteronuclear coupling

the observation of weak peaks, which are usually the signals of interest. The easiest solvent-suppression method uses a low-power saturation pulse applied at the solvent (H_2O in most cases) peak frequency for 1 to 2 sec prior to the 90º observation pulse to reduce the solvent peak. Other more sophisticated solvent-suppression techniques can also be used.[11–13]

In many cases, the analytical tasks are simply to detect and quantify a specific known analyte. Examples include the detection and quantification of commonly used buffer components (e.g., Tris, acetate, citrate, MES, propylene glycol, etc.). These simple tasks can readily be accomplished by using a standard one-dimensional NMR method. In other situations, the analytical tasks may involve identifying unknown compounds. This type of task usually requires homonuclear and heteronuclear two-dimensional NMR experiments, such as COSY, TOCSY, NOESY, HSQC, HMBC, etc. The identification of unknown molecules may also require additional information from other analytical methods, such as mass spectrometry, UV-Vis spectroscopy, and IR spectroscopy.[14]

One-dimensional proton NMR spectroscopy is the most straightforward method for process validation and development. It can be used as a limit test, i.e., to demonstrate that a particular analyte is below the detection limit. It can also be used to accurately quantify an analyte by comparing the NMR peak area from a test sample against a standard curve. To get accurate quantitation, it is important to keep the acquisition parameters and conditions constant for both standard and test samples. For example, the receiver gain, power level, and duration of all pulses must stay the same within an assay. In addition, the probe should remain tuned for all samples.

For biopharmaceutical process validation, it is often necessary to detect and quantify small molecules in the presence of large protein molecules. A standard CPMG spin-echo experiment[15,16] may be used for this purpose. The spin-echo method reduces the broad signals, which arise mostly from large molecules such as proteins, while it preserves the sharp signals, which arise mostly from small molecules. If spin-echo cannot satisfactorily reduce the protein signals, removing the protein from the sample by ultrafiltration is an easy alternative. However, extra care must be taken to ensure that ultrafiltration does not introduce any impurities or remove analytes of interest. The ability to analyze small molecules either in the presence of protein or after a simple filtration step to remove protein is essential in the purity test for a biopharmaceutical product.

Because the sensitivity of NMR is the highest for protons compared to other nuclei, all examples of quantitation work described in this chapter are based on proton NMR data. The signals from other NMR active nuclei such as ^{19}F or ^{13}C may also be used for quantitation. The quantification of TFA using ^{19}F NMR is a good example. However, except for ^{19}F, the sensitivities and detection limits are usually compromised in these measurements because nuclei other than ^{1}H and ^{19}F typically have a lower natural abundance and a lower magnetogyric ratio.

ADVANTAGES AND DISADVANTAGES OF NMR FOR BIOPHARMACEUTICAL PROCESS DEVELOPMENT AND VALIDATION

One major advantage of NMR over other types of spectroscopy is that NMR signals are highly specific and quantitative. Because the resonance frequency

(chemical shift) of a nucleus is closely related to its local environment in a molecule and the structure of that molecule, each compound will have a unique set of NMR signals. In addition, the peak intensity of an NMR signal is linearly proportional to the population of the nuclei that contribute to that signal. This is true not only for the nuclei from the same molecule, but also for the nuclei from different molecules. The relative peak intensities of the NMR signals from the same molecule directly reflect the relative numbers of each type of nucleus in the molecule. In essence, NMR is a way to obtain a relative nuclei count for a molecule. It is also valid to compare intensities of NMR peaks arising from different molecules. Therefore, NMR offers a convenient and nondestructive way of obtaining relative concentrations of impurities, isomer ratios, etc. If accurate standards can be prepared, it is very straightforward to obtain absolute concentrations for a given analyte in solution. The specific and quantitative characteristics of NMR spectroscopy make it a very useful technique in quantitative analytical chemistry.

There are many other advantages of using NMR as an analytical method for process development or validation. For example, sample preparation required for NMR analysis is usually minimal. There is no need to perform buffer exchange or complex chemical reaction (e.g., chemical labeling) prior to NMR measurement. In addition, there is very little restriction on the nature of samples that can be analyzed by NMR. For proton NMR (most commonly used) analysis in aqueous solution, the only requirement is that the molecules to be analyzed by NMR must contain nonexchangeable protons. NMR measurement usually is compatible with a wide range of solvents (e.g., organic solvents, aqueous buffers, buffers containing high concentration of salt, etc.). These advantages significantly reduce the assay development time.

Like all analytical techniques, NMR spectroscopy also has its weakness. The major limitation is its sensitivity. For proton (the most sensitive nucleus) NMR, the detection limit is typically 10 to 100 μ*M* for small organic molecules. This detection limit can be achieved fairly easily within a reasonable experimental time at 500 MHz. However, NMR is still significantly less sensitive than other techniques such as mass spectrometry. In spite of this limitation, the proton NMR is still sensitive enough to detect small organic molecules at a level near 1 to 10 μg/ml, which is sufficient for process validation purposes in most cases. The sensitivity and resolution of NMR are also limited by the size of molecules. While NMR has routinely been used in studying structures of macromolecules such as polymers, proteins, DNA, and polysaccharides, the NMR signals arising from these large molecules are normally much broader and weaker. Therefore, structural studies of these molecules by NMR often require a high sample concentration (>1 m*M*). In extreme cases, the NMR signals of large molecules become so broad that they cannot be observed even at a very high sample concentration. Quantitative applications of NMR are therefore primarily limited to small molecules.

CLEARANCE OF PROCESS-RELATED IMPURITIES

Trace levels of process-related impurities might have a profound effect on the quality and safety of a biopharmaceutical protein product. Therefore, the process validation study must demonstrate the removal of these impurities to an acceptable level by the recovery process. Detecting a low-level (<10 μg/ml) small-molecule impurity in the presence of active protein and excipients at much higher concentrations is a daunting analytical problem. In many cases, NMR is an excellent method for solving such challenging problems. The main advantage of using NMR is that the final formulated protein sample can be analyzed directly by NMR. The ability of detecting process buffer components in a formulated protein product by proton NMR is illustrated by a few examples described below. The same method can easily be applied to quantitation of other small proton-containing molecules.

Trace A in Figure 12.1 is a proton NMR spectrum of a control sample containing a mixture of three process buffer components (succinate, TEA, and TMA) in sodium acetate, a formulation buffer for a recombinant protein. The proton NMR spectrum in Figure 12.1 (trace A) shows that the signals of the three process buffer components are all unique and well resolved from each other. These unique signals from different molecules demonstrate that simultaneous detection of multiple analytes in a single spectrum is possible if the signals from those analytes do not overlap. Traces B and C are the proton NMR spectra of the formulated recombinant protein (2.5 mg/ml protein in sodium acetate buffer) with and without the spike of the three process buffer components (10 μg/ml each). Because the protein in this sample is a large molecule (MW > 25,000), the protein signals appear in the spectrum as very broad and weak peaks over a large spectral width. These broad and weak protein signals usually give rise to a rough or wavy baseline in the spectrum (trace B in Figure 12.1). This type of baseline makes the detection and quantification of low-level small-molecule impurities difficult. However, the protein signals can be minimized by the spin-echo method (traces D and E in Figure 12.1) to allow for better detection and quantification of small molecules. The NMR spectra in Figure 12.1 clearly indicate that TEA and TMA are not present in this formulated protein sample, whereas a small amount of succinate is present. The level of succinate in the final formulated protein sample was determined to be about 12 μg/ml based on its peak area and a standard curve.

Because NMR can be used to detect and quantify process buffer components, it is a convenient method to monitor clearance of the penultimate buffer during the final diafiltration step of a purification process. An example is shown in Figure 12.2. In this case, a protein was initially in a buffer containing several components including Tris, HEPES, and citrate (top spectrum). The buffer was exchanged during the diafiltration with 10 m*M* histidine (the diafiltration buffer). After 10 diavolumes, the Tris and HEPES are reduced below the limit of quantification, and the residual citrate level is 64 μg/ml. This example demonstrates

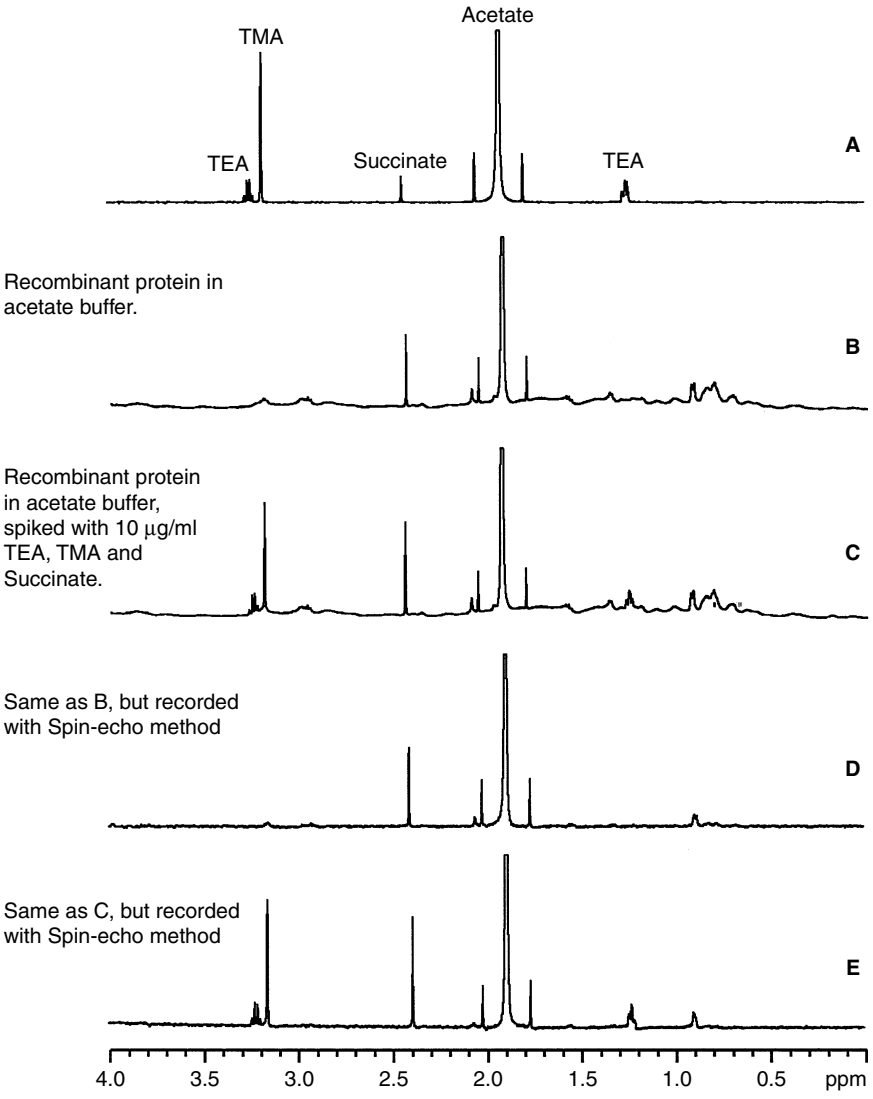

Figure 12.1 Clearance of small-molecule impurities from process buffers in a formulated protein product. Trace A: the NMR spectrum of a control sample containing a mixture of three components (succinate, tetraethylammonium, and tetramethylammonium) in the final formulation buffer (sodium acetate). These three components were used in the recovery process for a biopharmaceutical product. Traces B and D: the proton NMR spectra of the formulated protein product. No TEA or TMA were detected, but a small amount of succinate was observed in this sample. Traces C and E: the proton NMR spectra of a formulated protein product spiked with 10 μg/ml of succinate, TEA, and TMA. Traces D and E were recorded with CPMG spin-echo method to reduce the protein signals. The reduction of NMR signals from the protein allows for better observation of the small-molecule signals.

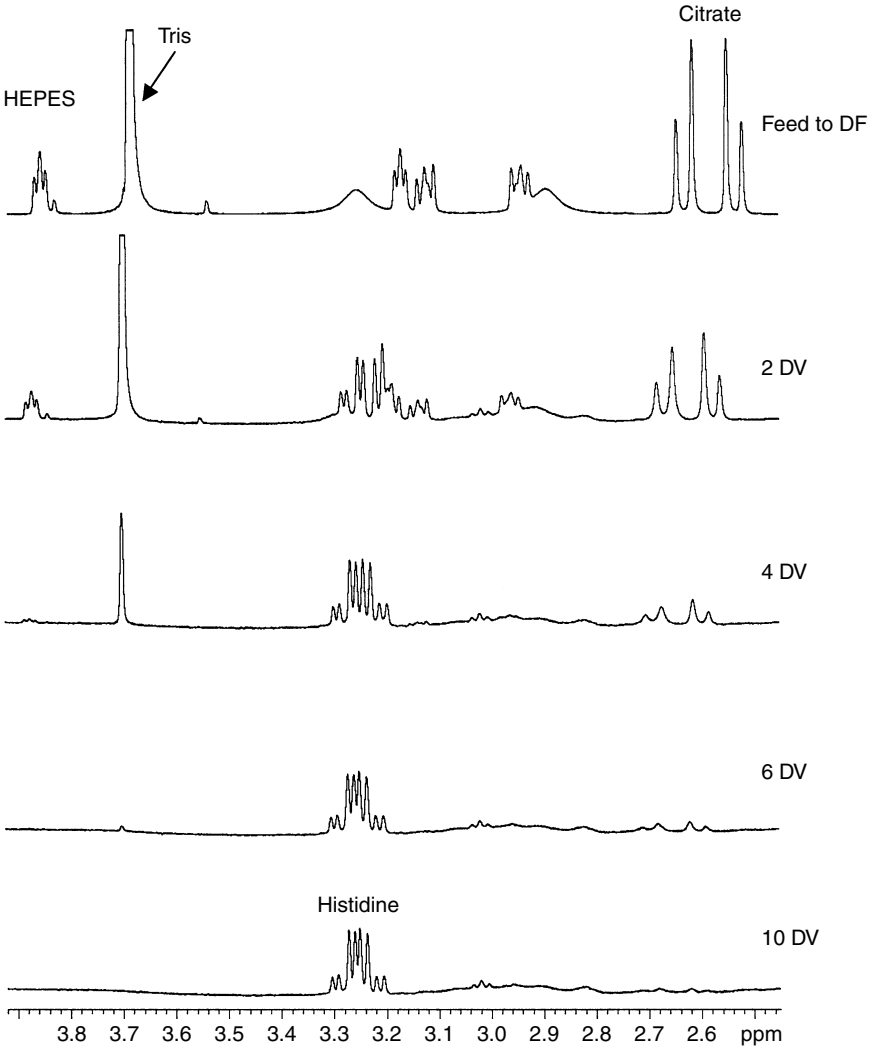

Figure 12.2 Using NMR to monitor the clearance of penultimate buffer components by diafiltration. The traces from top to bottom are the NMR spectrum of the feed to the dialfitration and the spectra of the samples taken after different diavolumes of buffer exchange have been completed. The penultimate buffer components (including HEPES, Tris, and citrate) are clearly removed after 10 diavolumes of buffer exchange.

that the NMR method can be used to verify and validate the performance of the diafiltration operation.

In some cases, the NMR signals of a small-molecule analyte might be broad and poorly resolved because of a chemical exchange process, for example,

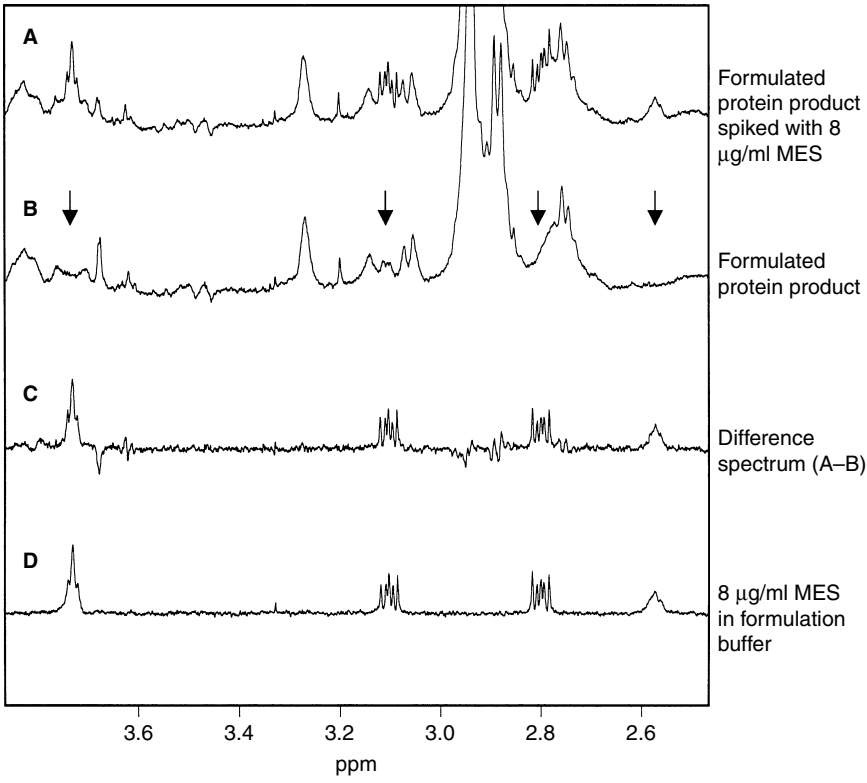

Figure 12.3 Clearance of MES in a formulated protein product. Trace A: the proton NMR spectrum of a formulated protein product spiked with 8 μg/ml of MES. Trace B: the proton NMR spectrum of a formulated protein product. The arrows indicate the positions where MES signals would be detected if present. Trace C: the difference of traces A and B (A-B). Trace D: proton NMR spectrum of 8 μg/ml of MES in the formulation buffer. The NMR spectra in traces A, B, and D were recorded with the CPMG spin-echo method to reduce protein signals. Only the region where MES signals appear is shown.

exchanging between two protonation states or two different conformational isomers. The spin-echo method will not work well in this situation. However, by carefully inspecting the spectrum one may still unambiguously determine if the analyte is present in the protein product. Figure 12.3 illustrates an example of detecting MES in a recombinant protein product by NMR. Trace D of Figure 12.3 is a proton NMR spectrum of MES (8 μg/ml) in the formulation buffer. At the pH of formulation buffer, the MES is in exchange between different protonation states. As a result, the NMR signals of MES are broader than those arising from other molecules with a similar size. These broad NMR signals make the

detection of MES in the formulated protein sample (trace B) somewhat troublesome. However, by carefully comparing the NMR spectrum of the formulated protein sample spiked with 8 μg/ml of MES (trace A) with the spectrum of the nonspiked sample (trace B), one concludes that the MES signals are absent in the spectrum of formulated protein sample (trace B). Subtracting the spectrum of nonspiked sample from the spectrum of the spiked sample leads to full recovery of the NMR signals of 8 μg/ml MES (trace C).

Another way to detect small molecules in the final formulated protein product without the interference from the protein signals is to remove the protein by ultrafiltration. Figure 12.4 compares a section of the proton NMR spectra of a biopharmaceutical protein product before (upper spectrum) and after (bottom spectrum) the protein was removed by ultrafiltering the sample with a Centricon-10 (Millipore Corp, Bedford, MA). Removing protein results in a flatter baseline (bottom spectrum). If small molecules are present in a protein sample, the removal of the protein may allow for unobstructed detection of the small molecules. In this case, a small amount of acetate (~ 1 μg/ml) is detected in the sample [bottom trace, Figure 12.4]. Figure 12.5 shows that spikes of 10 μg/ml of acetate and MES into the protein sample are fully recovered after the ultrafiltration to remove the protein. This example demonstrates that the interference of protein with the detection and quantitation of small-molecule impurities in a formulated protein product can be effectively eliminated by ultrafiltration.

Removing the protein offers an opportunity to detect not only process-related impurities, but also any small-molecule impurities. In fact, NMR is perhaps the easiest way to quickly determine if any significant amounts of small-molecule impurities are present in the final bulk of a protein product. Because sharp lines from small organic compounds can be readily detected at levels of 1 to 10 μg/ml and above, their absence in the NMR spectrum provides compelling evidence for the clearance of low-molecular weight impurities. Figure 12.6 displays two sections of the proton NMR spectrum for a protein product recorded after the protein has been removed. After the removal of the protein, only the formulation buffer (25 m*M* citrate) and a small amount (~1 μg/ml) of residual Tris are observed. For this product, Tris is a component of the penultimate buffer used in the recovery process prior to the final UF/DF formulation step. The Tris signal has a signal-to-noise ratio of about 70, indicating that NMR sensitivity is more than enough to detect small-molecule impurities at 1 μg/ml. Moreover, the absence of any other significant proton NMR signals in Figure 12.6 provides convincing evidence that other small-molecule impurities are unlikely to be present at levels greater than 1 μg/ml.

The removal of protein by filtration is a convenient way to avoid the interference of protein in the NMR measurement. However, this method requires that the analyte is not physically associated with the protein; otherwise, ultrafiltration will remove the analyte along with the protein. In addition, extractables from the devices employed to remove the protein may be introduced into the sample. Therefore, proper controls must be prepared and analyzed. Despite these

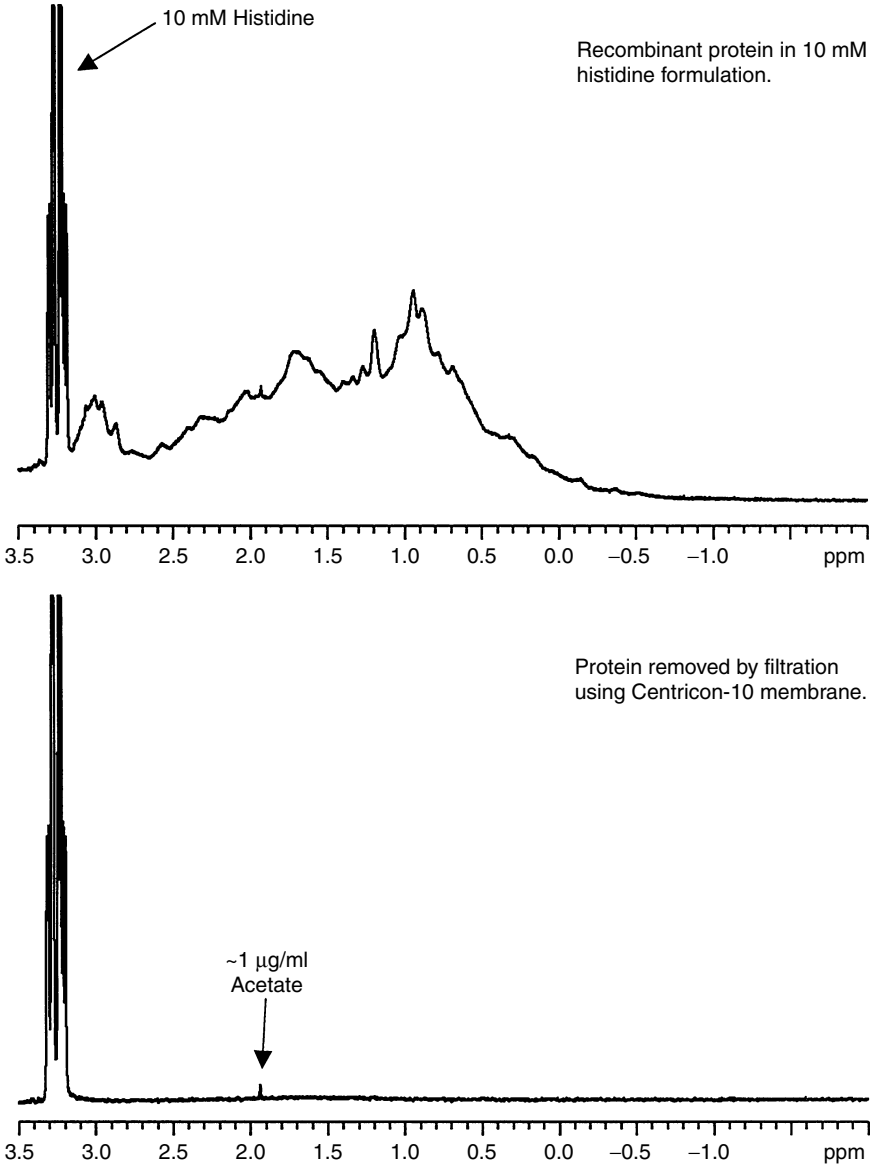

Figure 12.4 A section of the proton NMR spectra of a biopharmaceutical protein product before (upper spectrum) and after (bottom spectrum) the protein was removed by ultrafiltering the sample with Centricon-10.

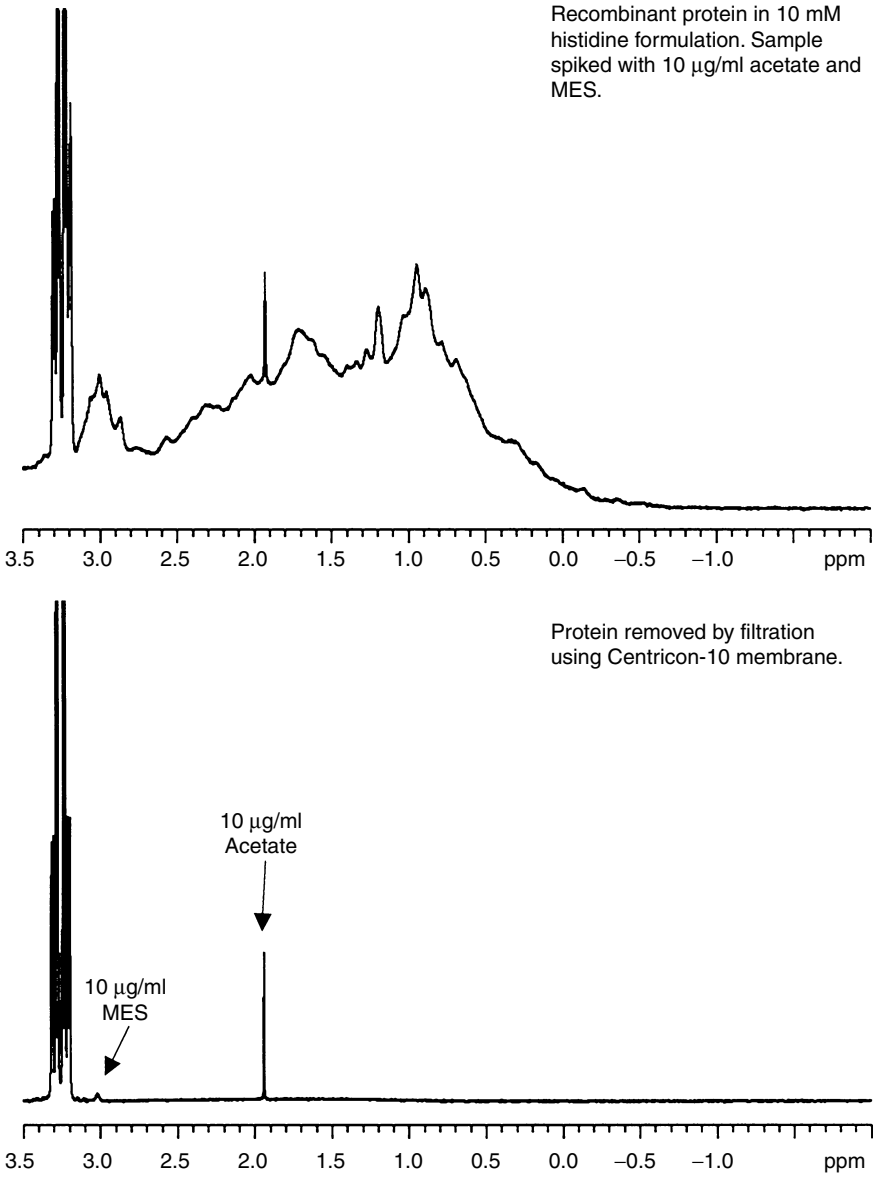

Figure 12.5 A section of the proton NMR spectra of a biopharmaceutical protein product, spiked with10 µg/ml of acetate and MES, before (upper spectrum) and after (bottom spectrum) the protein was removed by filtering the sample with Centricon-10. The acetate and MES are recovered after the filtration to remove the protein.

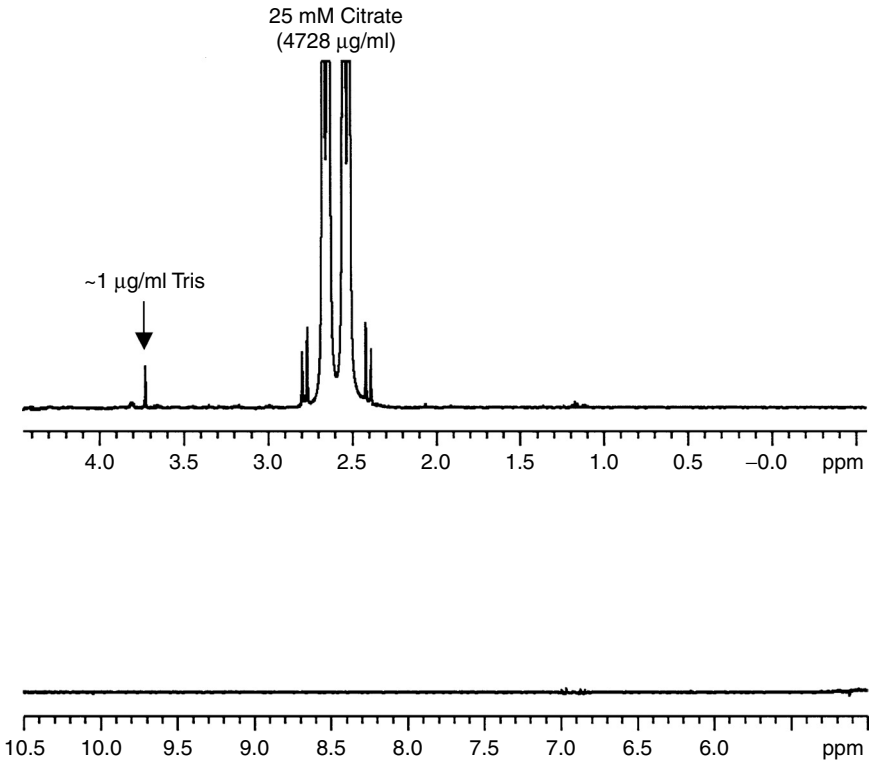

Figure 12.6 Two sections of the proton NMR spectrum for a protein product after the protein has been removed. Only the formulation buffer (25 m*M* citrate) and a small amount (~1 μg/ml) of residual Tris (a component of the penultimate buffer used in the recovery process prior to the final UF/DF formulation step) are observed after the filtration. The signal-to-noise ratio of Tris is about 70. The absence of any other significant proton NMR signals provides compelling evidence for good clearance of any other small proton-containing molecule impurities.

potential problems, ultrafiltration of the sample prior to the NMR measurement is still a powerful method. In many cases, it can provide important data to complete the impurity profile for a biopharmaceutical product.

QUANTITATION OF WATER-SOLUBLE POLYMERS

Quantitative NMR analysis may also be applied to water-soluble polymers or copolymers in some cases. Polymers usually have large molecular weights and are heterogeneous in size. However, the proton NMR signals of some polymers

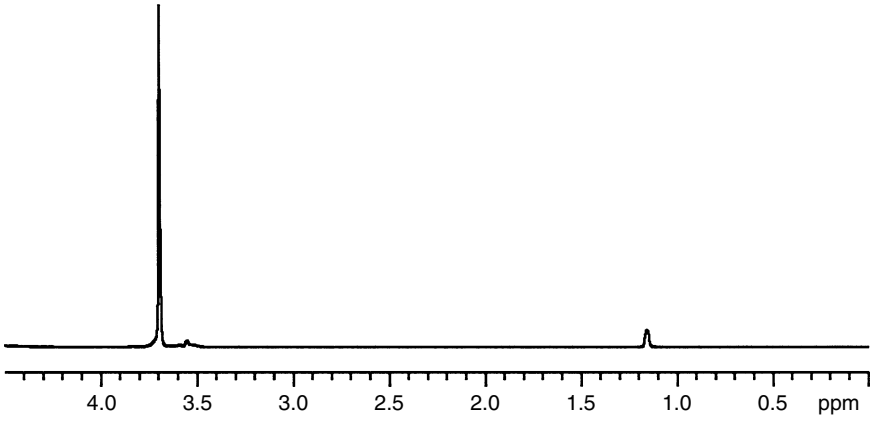

Figure 12.7 Proton NMR spectrum of a typical polymer used in mammalian cell culture processes: Pluronic F68.

are very simple and can be easily quantified by NMR. A good example is Pluronic F-68, an inhomogeneous polyethylene oxide/polypropylene oxide copolymer often used in cell culture media to reduce mechanical lysis of cells. The NMR spectrum of Pluronic F68 [Figure 12.7] contains relatively broad signals near 1.2 ppm (methyl protons) and between 3.5 and 3.9 ppm (methylene protons). The nonsymmetrical peak shape and the large line width of these signals are typical features of inhomogeneous polymers. The peak at about 3.7 ppm can be used to quantify Pluronic F-68 because it is the strongest signal. As demonstrated in Figure 12.8, a standard curve covering a range from 12.5 µg/ml to 50 µg/ml can easily be obtained for Pluronic F-68 in a buffer containing a significant amount of MOPS and acetic acid. Note that Pluronic F-68 may be detectable at a level lower than 12.5 µg/ml because the peak at 3.7 ppm still has a relative high signal-to-noise ratio (about 150) at 12.5 µg/ml. Normally, the purification process can clear the Pluronic F-68 to below this detection limit. The top spectrum in Figure 12.9 shows that the Pluronic F-68 signals are absent from the proton NMR spectra of in-process samples of a protein product. If Pluronic F-68 were not cleared, its NMR signal would have been detected, as clearly evidenced in the NMR spectrum of the in-process protein sample spiked with 12.5 µg/ml of Pluronic F-68 [Figure 12.9, bottom spectrum]. Because of the inhomogeneous nature of Pluronic F-68, this type of analysis cannot be easily done with other analytical techniques, such as HPLC or mass spectrometry. It is therefore important to include the NMR analysis in the process validation to demonstrate the clearance of Pluronic F-68. Quantitation of other polymers such as polysorbate 20, polyethylene glycol, etc., by NMR can also be achieved.

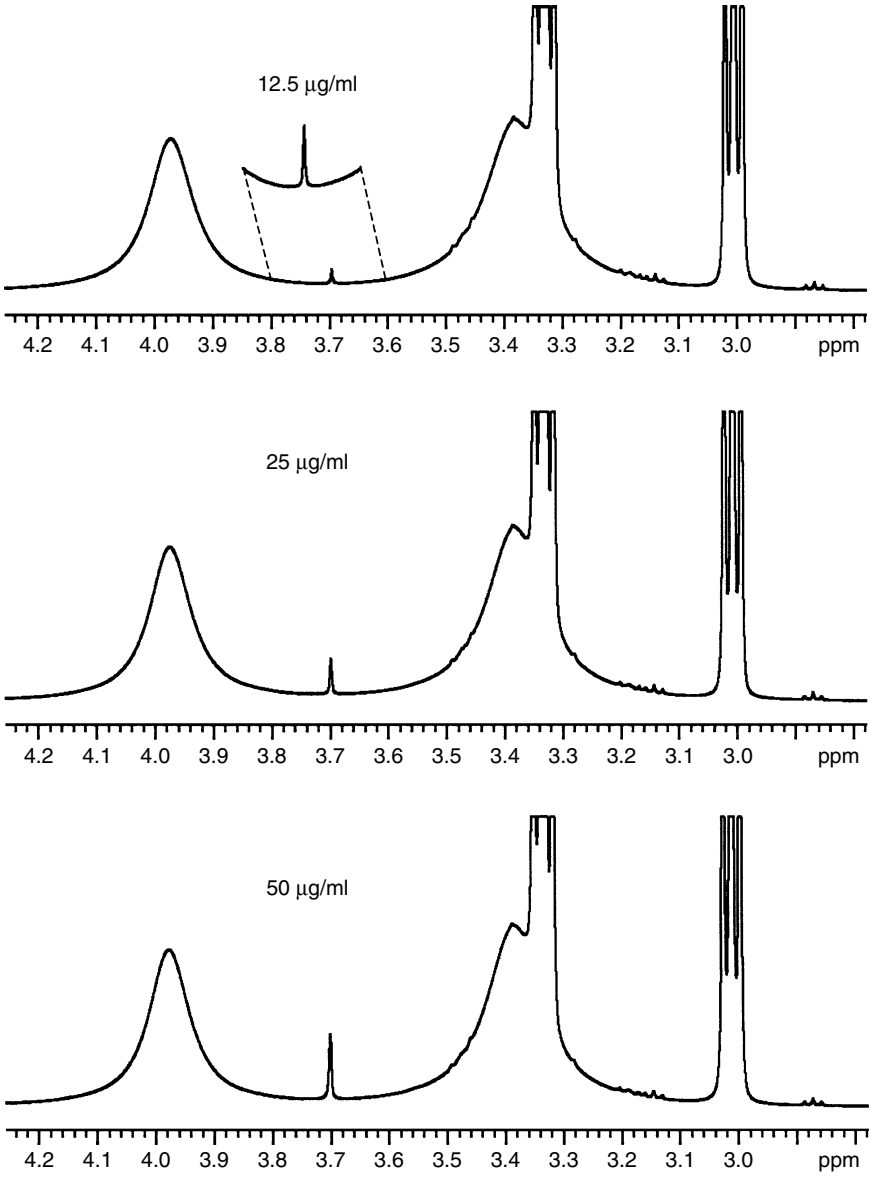

Figure 12.8 Proton NMR spectra of standards of Pluronic F68 prepared in a process buffer covering a concentration range from 12.5 μg/ml to 50 μg/ml.

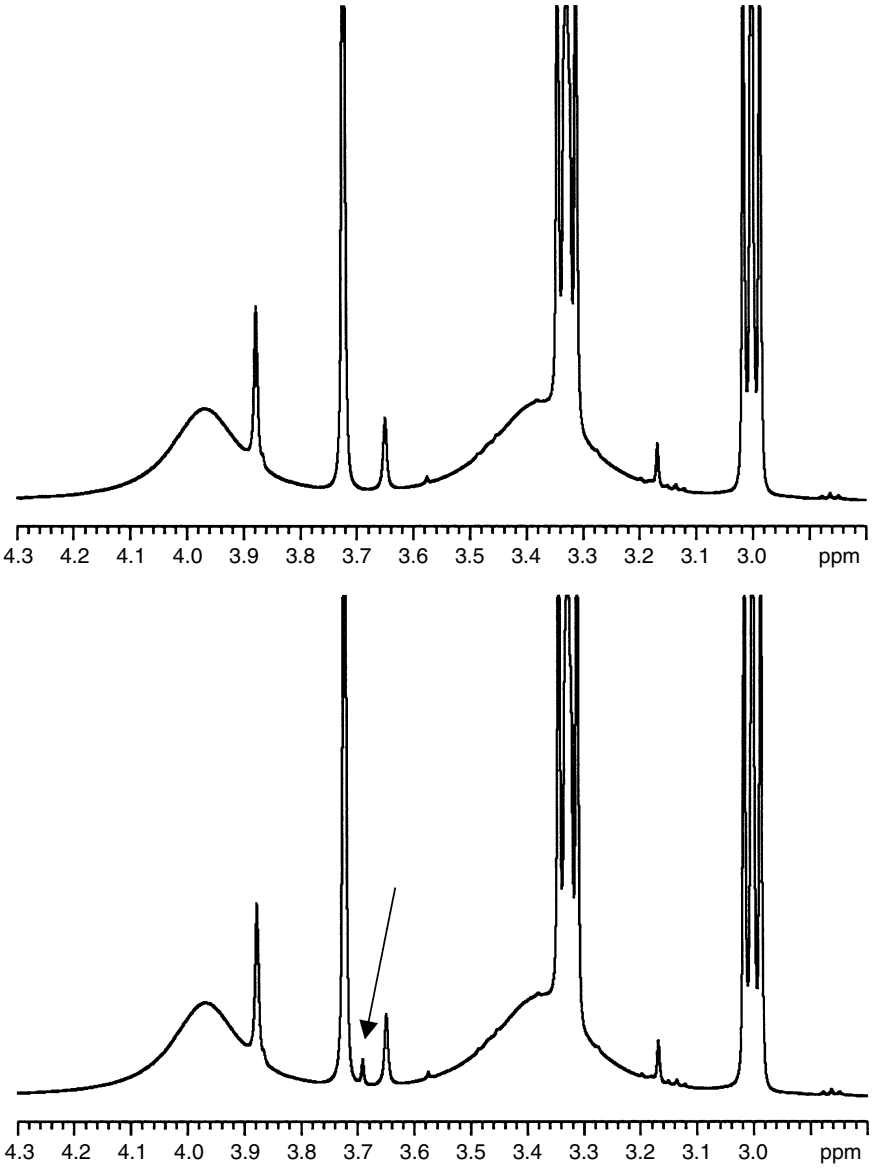

Figure 12.9 Proton NMR spectra for an in-process sample of protein product with (bottom spectrum) and without (upper spectrum) a spike of 10 μg/ml Pluronic F-68. The Pluronic F-68 signal is clearly absent in the upper spectrum. This example demonstrates that if the Pluronic F-68 level was greater than 12.5 μg/ml, it would have been observed.

STRUCTURAL CHARACTERIZATION BY NMR

In order to successfully obtain regulatory approval for a biopharmaceutical product, detailed product characterization is essential. Typically, mass spectrometry coupled with separation methods such as LC or CE can be effectively used for characterization purposes. Even though NMR has been used extensively for the structural characterization of proteins, obtaining a three-dimensional protein structure using NMR is not a trivial task. It requires isotope enrichment of the protein, complex multidimensional NMR experiments, and sophisticated structural computation. Structural determination by NMR is routinely done only for small proteins (<25 to 30 kDa). Currently, it is not the best method for studying the structures of very large proteins like full-length antibodies. However, it is still a useful method to aid the characterization of a biopharmaceutical product in many cases. For example, NMR has been used to characterize the structures of carbohydrates in glycoproteins[17–19] and to identify the sites of deamidation within a protein.[20]

NMR can also be used to elucidate the structural features of a repeating unit in a polysaccharide and to investigate the conformation and dynamics of polysaccharides.[21] A unique polysaccharide structure results in a characteristic proton NMR spectrum. Therefore, NMR is a powerful tool for identifying polysaccharide structures. This remarkable specificity has led to the development of a routine NMR-based identity assay, recently reported by Abeygunawardana et al. for quality control testing of bacterial polysaccharide to be used in formulating a polyvalent pneumococcal polysaccharide vaccine.[22]

Because NMR is a very versatile technique and can be used more effectively for structural characterization, it is possible to find many other applications for it in biopharmaceutical product characterization. For example, it can be used to determine the structures of small organic molecule impurities. Additionally, it is possible to apply NMR to study structures of peptide fragments obtained from peptide mapping of a biopharmaceutical product. Peptide mapping is often a good method to study the variants and degradation product of a protein. However, it is necessary to employ additional methods to determine the structure of each peptide in a map. The combination of peptide map, mass spectrometry, and NMR may provide a great deal of structural detail of a biopharmaceutical protein product. More applications of NMR to the characterization of a biopharmaceutical product are likely to emerge when it becomes a more general and readily available technique.

VALIDATION OF NMR ASSAY

Validation is critical for any assays to be used in pharmaceutical development. Validating an NMR assay is fairly straightforward. Some validation characteristics for an NMR assay are briefly discussed in this section.

Specificity: NMR spectroscopy is a specific analytical method by its nature. Each type of proton located in a different chemical or magnetic environment in the same or different molecule can be differentiated through its characteristic resonance frequency (Figure 12.1A). Thus, every proton-containing chemical has a unique set of proton NMR signals that can be readily identified. Although the NMR signals may be dependent on the solvent conditions (for example, temperature, pH, hydrophobicity, etc.), each molecule still can be uniquely identified in an NMR spectrum.

Linearity and range: Linearity is an inherent property of NMR spectroscopy. A standard curve can be easily obtained to cover a wide range of concentrations with a typical R^2 value of >0.99.

Precision and accuracy: Quantitative analysis by NMR is very precise with relative standard deviations for independent measurements usually much lower than 5%. The largest errors in NMR measurements are likely due to sample preparation, not the NMR method itself. If a good set of standards is available and all NMR measurements for the test and standard samples are performed under the same acquisition conditions, the quantitative results can be readily reproduced on different instruments operated by different analysts at different times. Therefore, good intermediate precision can also be achieved. An accurate quantitative NMR assay will require accurately prepared standards. The accuracy of an NMR assay can be assessed, for example, by measuring an independently prepared standard or an accurate reference sample with the assay. In many cases, a spike recovery experiment can also be used to demonstrate the accuracy of an NMR assay.

Detection and quantitative limit: Unless the NMR signals from the analyte are obscured by the NMR signals from buffer components, the detection limit of NMR measurement is typically 1 to 10 μg/ml for small organic molecules. The actual detection limit for an analyte may vary depending on the acquisition parameters and instrument settings. In particular, the more transients that are used for acquisition, the higher the sensitivity. Therefore, increasing the acquisition time can lower the detection limit. A signal-to-noise ratio of >5:1 for the signal of interest can be used to define the detection limit. A detection limit of 1 to 10 μg/ml for small organic molecules is probably sufficient for the detection of nontoxic impurities. Although this detection limit is not extraordinary, the ability of demonstrating that no small-molecule impurities are present in a protein product at a level above 1 to 10 μg/ml with a single NMR spectrum is, in fact, very valuable.

The quantitation limit is the lowest concentration of analyte in the standard curve with a signal-to-noise ratio of at least 10. Typically, the quantitation limit for most small organic molecules is 10 μg/ml. If necessary, the quantitation limit can be lowered, as long as the acquisition parameters are adjusted to yield sufficient sensitivity.

Robustness: Even if the NMR instrument is not properly calibrated (for example, the probe tuning and pulse length calibration are not optimized), as

long as the acquisition parameters are kept constant during the assay, high precision and accuracy can still be achieved. In addition, the relative resonance frequencies (chemical shifts) of NMR signals do not depend on the experimental parameters; therefore, the spectrum of the same sample recorded using different acquisition parameters by different operators will contain the same peaks with the same chemical shifts and the same relative intensities. This significantly reduces the variations arising from instrumentation and acquisition parameters. Thus, the NMR assay is a very robust quantitative assay.

Suitability: The main requirement for the NMR measurement to be suitable for quantitating small organic molecules is that the NMR peaks of analyte must be resolved from any background signals (for example, from buffers or solvents). In some cases, sample conditions must be changed in order to resolve the peak of interest. For example, the Tris signal (a singlet at 3.7 ppm) is pH dependent. By changing the solution pH, one can move the Tris signal to a position where background signals do not interfere with the quantitation. As a result, the suitability must be evaluated individually for each analyte. However, the NMR assay normally can be qualified for the quantification of most small organic molecules without difficulties.

CONCLUDING REMARKS

NMR is a remarkably flexible technique that can be effectively used to address many analytical issues in the development of biopharmaceutical products. Although it is already more than 50 years old, NMR is still underutilized in the biopharmaceutical industry for solving process-related analytical problems. In this chapter, we have described many simple and useful NMR applications for biopharmaceutical process development and validation. In particular, quantitative NMR analysis is perhaps the most important application. It is suitable for quantitating small organic molecules with a detection limit of 1 to 10 μg/ml. In general, only simple one-dimensional NMR experiments are required for quantitative analysis. The other important application of NMR in biopharmaceutical development is the structural characterization of molecules that are product related (e.g., carbohydrates and peptide fragments) or process related (e.g., impurities and buffer components). However, structural studies typically require sophisticated multidimensional NMR experiments.

There has been significant advancement in the applications of NMR to the development of small-molecule pharmaceutical products. For example, advances in NMR automation (e.g., flow-injection analysis) and directly coupled methods (e.g., LC-MS-NMR analysis) have made analysis and characterization of small-molecule drugs much easier.[23–25] These improvements have helped chemists to develop and characterize small-molecule combinatorial libraries and to screen for active compounds.[4–6] It is likely some of these techniques can also be used in biopharmaceutical product development.

NMR offers many unique advantages that other methods cannot provide in spite of some limitations. Biopharmaceutical product development will certainly benefit from including NMR as an option for solving analytical problems. NMR instrumentation and methodology are constantly being improved. As better and more sensitive NMR techniques become available, the use of NMR as a standard analytical tool in biopharmaceutical process development and validation is expected to increase.

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