Assessment of Higher Order Structure Comparability in Therapeutic Proteins Using Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: In this work, we applied nuclear magnetic resonance (NMR) spectroscopy to rapidly assess higher order structure (HOS) comparability in protein samples. Using a variation of the NMR fingerprinting approach described by Panjwani et al. [2010. J Pharm Sci 99(8):3334–3342], three nonglycosylated proteins spanning a molecular weight range of 6.5–67 kDa were analyzed. A simple statistical method termed easy comparability of HOS by NMR (ECHOS-NMR) was developed. In this method, HOS similarity between two samples is measured via the correlation coefficient derived from linear regression analysis of binned NMR spectra. Applications of this method include HOS comparability assessment during new product development, manufacturing process changes, supplier changes, next-generation products, and the development of biosimilars to name just a few. We foresee ECHOS-NMR becoming a routine technique applied to comparability exercises used to complement data from other analytical techniques. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:1724–1733, 2013

Keywords: Higher order structure comparability; biopharmaceuticals characterization; NMR; spectroscopy; ECHOS-NMR; NMR fingerprinting; proteins; protein structure; diffusion

INTRODUCTION

An assessment of comparability in biological products after introduction of manufacturing changes[1,2] or for licensing purposes of biosimilars[3] is essential for the demonstration of quality, safety, and efficacy. Physicochemical characterization is a key component of the comparability exercise and, in some cases, this alone can help avoid costly preclinical or clinical studies. Analytical techniques such as mass spectrometry, capillary electrophoresis, size-exclusion chromatography (SEC), circular dichroism (CD), Fourier transformed infrared spectroscopy (FT-IR), fluorescence, ultraviolet (UV) spectroscopy, and peptide mapping are commonly used.[4,5] For protein products, these techniques provide information about their amino acid sequence and modifications, mass, size, charge, aggregation, glycosylation, and secondary structure. Information about the tertiary and quaternary structures can be obtained indirectly through functional assays or directly by near-UV CD, nuclear magnetic resonance (NMR) spectroscopy, and X-ray crystallography when the molecule is amenable to crystallization.

Optical spectroscopic techniques such as CD and FT-IR have been used extensively in the biopharmaceutical industry to characterize protein structure in comparability exercises. However, with the ever-increasing need to better understand the product, orthogonal analytical techniques that can probe for higher order structure (HOS) should be implemented. NMR spectroscopy is ideally suited for such analyses. In NMR, the resonance frequency (or chemical shift) of a given atom depends on the surrounding electronic environment. Therefore, the chemical shifts of NMR-active nuclei (such as protons, carbons, and nitrogens) are dependent on the molecule's three-dimensional structure. NMR spectra are thus structural fingerprints representing a protein's HOS and can be compared to assess similarity between samples.

Protein NMR has traditionally been used for structural and dynamic characterizations of isotopically enriched polypeptides.[6] Isotopic labeling is generally not feasible for biopharmaceuticals; however, recent

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advances in instrument sensitivity have made it possible to analyze proteins at natural abundance and low concentrations. Despite the promising potential of NMR as an orthogonal analytical technique for HOS comparability studies of therapeutic proteins, only a few examples can be found in the literature. The use of two-dimensional (2D) $^{1}H/^{1}H$-nuclear overhauser effect spectroscopy (NOESY) spectra has been proposed for characterization of the structure and consistency of manufacturing[7] and to compare the structural similarity of a protein in solution from different preparations.[8,9] NMR fingerprinting using $^{1}H/^{15}N$-heteronuclear single quantum correlation (HSQC) spectra has been used to assess the identity of the bioactive conformation for recombinant human granulocyte macrophage-colony stimulation factor (rhGM-CSF).[10,11] More recently, solid-state NMR spectra of lyophilized bovine pancreatic trypsin inhibitor (BPTI) and insulin samples have been developed and used for structural comparisons.[12] One-dimensional (1D) proton NMR was used as part of the comparability exercise for the biosimilar Zarzio® (filgrastim) (Sandoz GmbH, Kundl, Austria).[13]

Here, we explore the potential of NMR for the HOS comparability in therapeutic proteins. First, we examined the effects of a source change in a next-generation product by assessing the chemical shift differences of assigned peaks. Alternatively, we analyzed the NMR data with easy comparability of HOS by NMR (ECHOS-NMR). This method couples the NMR fingerprinting principle[11] with a simple statistical analysis to easily quantify the degree of structural similarity. Second, using ECHOS-NMR, we compared the HOS of an active pharmaceutical ingredient (API) from a different supplier to that of a reference-listed drug (RLD). And third, ECHOS-NMR was used to examine the effects of a manufacturing process and a variation of this process on the structural integrity of a protein. In addition to the HOS comparability assessment by ECHOS-NMR, we also propose the use of NMR-derived diffusion coefficients (*D*s) to further characterize the structural similarity between samples.

EXPERIMENTAL

NMR Samples

Bovine Pancreatic Trypsin Inhibitor

Synthetic and bovine-derived proteins were equilibrated under conditions that mimicked the finished drug product, the buffer for which included sodium citrate and histidine. The protein solution was further dialyzed against this product buffer using a 3500 molecular weight cut off regenerated cellulose tube (Spectra/Por, Spectrum Laboratories, Inc., Rancho Dominguez, California), then dialyzed against water, and finally lyophilized. Test solutions were prepared by dissolving 30 mg of the lyophilized protein in approximately 500 μL of D_2O or H_2O/D_2O (90:10). Final concentration was approximately 30 mg/mL (~5 mM).

API and RLD

Test solutions of the API were made by dissolving the lyophilized protein in solution conditions mimicking the RLD matrix. Five hundred microliters of the protein solution was mixed with 50 μL D_2O. RLD solutions were prepared by aliquoting 500 μL from the drug vial and adding 50 μL D_2O. The final protein concentration was approximately 4 mg/mL (~0.6 mM). Disulfide bond reduction was accomplished by the addition of incremental amounts of a 100 mM 2-mercaptoethanol (BME) in D_2O stock solution to an RLD sample. There were three moles of disulfide bonds per mole of RLD. Solutions with estimated 6, 28, 50, and 100 mol % of reduced disulfides were made.

Albumin

Test solutions were made by dissolving 50 mg of lyophilized protein in 1 mL of D_2O (~0.7 mM).

NMR Spectroscopy

Bovine Pancreatic Trypsin Inhibitor

One-dimensional ^{1}H and a series of 2D homonuclear ^{1}H spectra were acquired including total correlation spectroscopy (TOCSY), NOESY, and double-quantum filtered correlation spectroscopy (DQF-COSY). All data were obtained on a 600 MHz Bruker DRX NMR spectrometer (Bruker Corporation, Billerica, Massachusetts) with a triple resonance inverse probe, and the sample temperature was maintained at 37°C. Felix 2000.1 (Felix NMR, Inc., San Diego, California) was used for data processing and chemical shift assignments. Chemical shift assignments were based on TOCSY, NOESY (300 ms mixing time), and DQF-COSY experiments.

API, RLD, and Albumin

Data were acquired on a 600 MHz Bruker AVANCE III NMR spectrometer equipped with a Dual-$^{13}C/^{1}H$ cryoprobe. The sample temperature was maintained at 40°C during acquisition of the 1D ^{1}H, ^{13}C, and 2D $^{1}H/^{13}C$- HSQC datasets. Diffusion data were obtained at 25°C. Spectra of materials from disulfide bond reduction experiments performed on the RLD were acquired at 25°C on an 850 MHz Bruker AVANCE III NMR spectrometer equipped with a QCI cryoprobe. TopSpin 3.0 software (Bruker Biospin, Rheinstetten, Germany) was used for data acquisition and processing.