Qualification of FTIR Spectroscopic Method for Protein Secondary Structural Analysis

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ABSTRACT: Fourier transform infrared (FTIR) spectroscopy is widely used to study protein secondary structure both in solution and in the solid state. The FTIR spectroscopic method has also been employed as a characterization method by the biopharmaceutical industry to determine the higher order structure of protein therapeutics, and to determine if any changes in protein conformation have occurred as a result of changes to process, formulation, manufacture, and storage conditions. The results of these studies are often included in regulatory filings; when comparability is assessed, the comparison is often qualitative. To demonstrate that the method can be quantitative, and is suitable for these intended purposes, the precision and sensitivity of the FTIR method were evaluated. The results show that FTIR spectroscopic analysis is reproducible with suitable method precision, that is, spectral similarity of replicate measurements is greater than 90%. The method can detect secondary structural changes caused by pH and denaturant. The sensitivity of the method in detecting structural changes depends on the extent of the changes and their impact on the resulting spectral similarity and characteristic FTIR bands. The results of these assessments are described in this paper. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:4631–4641, 2011

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INTRODUCTION

Fourier transform infrared (FTIR) spectroscopy is an absorption spectroscopy that can be used to obtain information about the vibrational states of molecules. The FTIR spectrum of a protein is composed of many vibrational bands arising from different functional groups such as N—H, C=O, and so on. The protein backbone amide groups generate a number of characteristic IR bands that can be used to determine protein backbone conformation and secondary structure. In particular, the amide I band, which is in the region of 1600–1700 cm^{-1}, is primarily due to the amide C=O stretching vibrations. Different secondary structures, such as alpha-helix, beta-sheet, and beta-turn, exhibit characteristic frequencies and intensities in the amide I band region due to differences in the hydrogen bonds in these structures.[1,2] Therefore the FTIR spectrum of a protein is routinely used to determine and characterize protein secondary structures in solution as well as in the solid state.[3–5]

Many attempts have been made to quantify the percentage of different secondary structural components for proteins in solution by FTIR and CD (circular dichroism); these results are compared with those obtained by X-ray crystallography in order to assess their accuracy.[1,6–8] For FTIR, there are two primary ways of doing this. The first uses curve fitting of the second derivative or self-deconvoluted spectra to obtain the relative amounts of different types of secondary structure based on the band areas.[1,9]

Abbreviations used: FTIR, Fourier transform infrared; Gdn, guanidine hydrogen chloride; C3N, 20 mM sodium citrate with 140 mM sodium chloride buffer at pH 3.0.DTGD, Deuterated Triglycine Sulfate

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The second involves peak fitting of the nondeconvoluted and baseline corrected amide I bands, and then obtains the percentage of secondary structures by correlating with the shape and intensity using an interval partial least squares algorithm.[6] These methods are helpful in determining the primary secondary structure components and estimating their percentages in a protein. However, because the quantitation of the secondary structure composition can vary depending on the methods and parameters used and the process typically involves considerable mathematical manipulations, they are not routinely carried out for protein secondary structure assessment by the biopharmaceutical industry. Furthermore, these methods are focused on quantitation of the secondary structure composition of each individual protein, but are not optimized for assessing overall similarity of protein structure between samples from different processes, or formulations. In contrast, the focus of this work is to evaluate the precision and sensitivity of the FTIR method for biopharmaceutical applications in which a degree of similarity between the spectra obtained from two or more samples is often the desired result. The higher order structure of protein therapeutics is complex in nature; the correct three-dimensional structure is required for proteins to be able to carry out their function and remain stable. It can be affected by manufacturing processes including refolding from inclusion bodies,[10] low pH elution during chromatographic and viral inactivation steps,[11] and so on. To ensure the product quality of a protein therapeutic, its higher order structure needs to be assessed.[12–14] FTIR spectroscopy is one of the spectroscopic techniques that has been used routinely to assess the secondary structure of protein therapeutics with the data included in regulatory filings. However, the qualification of the FTIR method has mostly remained elusive, mainly due to the lack of a quantitative way to directly compare the FTIR spectra of different samples without curve fitting or self-deconvolution. For this type of determination, the assignment of particular secondary structure content to the protein is not important. The focus is instead on the ability to detect small changes in structure, regardless of the source of the change. At present, there is no consistent method applied to quantitatively determine the comparability of different proteins, or of different lots of the same protein, so visual comparisons of a sample spectrum to a reference have typically been used as a way to verify proper folding of the protein. It is not straightforward to apply principles from International Conference on Harmonization guidelines Q2 (R1) "Validation of Analytical Procedures" to FTIR analyses because the spectra are not quantitative without subsequent mathematical treatment. A few approaches (mathematical algorithms) have been explored to compare the FTIR spectra quantitatively,[15,16] including the correlation coefficient and area of overlap methods by Kendrick et al.[15] and Prestrelski et al.[16] Each one has strengths and weaknesses, which will be detailed in a separate paper. In this paper, we applied a quantitative function—OMNIC QC Compare[17]—to directly compare FTIR spectra and identified important performance characteristics for the qualification of the FTIR method.

To demonstrate that the FTIR method is suitable for its intended applications, that is, to analyze a protein therapeutic conformation and changes under various process-related conditions, and to ensure the proper folding of the protein during storage and delivery, the precision and sensitivity of the method were assessed.

The precision of the method was evaluated through a multisite/instrument and multi-analyst study, wherein the same data collection and analysis method were used. Proteins containing different types of secondary structure such as alpha-helical and beta-sheet were included to ensure that the precision assessment would apply to secondary structure analysis of all proteins, regardless of the specific structural type. However, most of the data were acquired using proteins with beta-sheet secondary structure because antibody and antibody fragment-based therapies are prevalent in the biopharmaceutical industry. In addition, interday repeatability and effect of protein concentration (±10% of the targeted concentration) differences on the precision of the method was also evaluated. Characteristic FTIR bands and overall spectral similarity of spectra collected on the same proteins were compared. The precision of FTIR analysis from this study can be used to define method performance parameters.

The sensitivity of the method was evaluated by comparing the spectrum of a native or control protein to that of the partially or fully unfolded protein after low pH or denaturant treatment and also by blending studies, wherein a native protein spectrum was mixed with that of a denatured protein and the resulting spectra were compared with that of the native protein. Standard curves were generated where the spectral similarity was plotted as a function of the percentage of the unfolded protein. Spectral similarity determined using the OMNIC QC compare tool was used to quantitatively assess the sensitivity of the FTIR method.

MATERIALS AND METHODS

Materials

Proteins 1–11 in Table 1 were produced at Amgen Inc. and were at least 98% pure by size-exclusion chromatography. They were kept in their stable storage