Time-Dependent Changes of Oxytocin Using ^{1}H-NMR Coupled with Multivariate Analysis: a New Approach for Quality Evaluation of Protein/Peptide Biologic Drugs

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A new method that combines ^{1}H-NMR and principal component analysis (PCA) was employed to obtain the quality evaluation of biopharmaceuticals, with regard to their quality, consistency, and differences in protein modification patterns. To assess the feasibility of the method, three ^{1}H-NMR spectra of oxytocin (OXT) were collected every 7 d (at Day 0, 7 and 14), and time-dependent changes in the spectra were found by PCA of the ^{1}H-NMR signals from 0.5—9.0 ppm, excluding the region around the water signal (4.6—5.0 ppm). Although the three OXT spectra seemed similar by simple visual inspection, time-dependent differences among the three spectra were clearly distinguished by a PCA scores plot. Peak changes indicating both OXT decomposition and the emergence of new OXT decomposition products within the timeframe of the experiment were also observed by a PCA loading plot. The results demonstrate that this method can evaluate the consistency of biopharmaceutical quality.

Key words quality evaluation; biologic drug; principal component analysis; ^{1}H-NMR; oxytocin

The biotechnology industry has grown significantly in the past decade and continues to grow at a rapid rate. Biopharmaceuticals such as oxytocin (OXT), insulin, and somatropin are large, complex molecules that are receiving increased attention as therapeutics in humans, particularly since this class of molecules can potentially exert pharmacological effects that are unattainable by synthetic chemical products. However, while biologics show great potential value in medicine, many technical hurdles must be overcome before such treatments are made practical. In particular, since biologic drugs are typically derived from living sources such as microorganisms, plants, or human or animal cells, the production and use of such material introduces certain hazards that are not presented by small molecule drugs manufactured through chemical synthesis.[1)] In addition, to fully understand the mechanism of action of a protein/peptide drug, not only the primary amino acid sequence but also the folding, post-translational processing, and multimerization properties of the biologic within the cell must be considered. Moreover, different cell types or cell growth conditions may yield different protein modification patterns, as well as different impurities, into the desired product.[2,3)] Therefore, all of these complications may potentially influence the intended pharmacological effect of biologic drugs. With respect to safety and efficacy concerns surrounding biologics, the consistency of biologics between production lots, including maintenance of tight quality control specifications, is an important consideration for manufacturing biologic drugs.

To date, many analysis methods, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE),[4,5)] capillary electrophoresis (CE),[6)] mass spectrometry (MS), tandem MS (MS/MS),[7)] liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC)[8] and nuclear magnetic resonance (NMR), are employed to evaluate physicochemical characteristics and purity, and thus determine the quality of biopharmaceuticals.[2,3,9,10)] However, characterizing the full complexity of biologics by present examination methods is still currently difficult. Therefore, new analytical techniques that provide more detailed evaluation of biologic quality are necessary. Among the various analytical techniques, we focused on NMR profiling as the most suitable tool for rigorous quality evaluation because this approach can provide structural information on all compounds contained in product lots, and can distinguish structural differences. Principal component analysis (PCA) is often useful for profiling and classifying sample groups, and to characterize the most effective valuables in separation compounds.[11,12)] Therefore, small differences in product quality, *e.g.* a structural change or the appearance of a decomposition product, are thought to be appropriately evaluated by the combination of PCA using multivariate statistics and ^{1}H-NMR.

Herein, we demonstrate that ^{1}H-NMR spectroscopy coupled with PCA can provide a molecular fingerprint to precisely characterize a specific protein/peptide, using the determination of time-dependent changes of OXT as an example of this method. The results suggest that this new methodology can be useful for the quality evaluation of a manufactured protein/peptide biologic drug.

Experimental

Chemicals and Reagents All reagents used for ^{1}H-NMR experiments were purchased from Wako Chemicals, were of analytical grade (purity >99%), and were used without further purification. Deuterium oxide (D_2O, isotopic purity 99.9%) containing 0.75% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) was purchased from Aldrich (St. Louis, MO, U.S.A.). TSP was used as an internal standard at a chemical shift (δ) of 0.0 ppm for ^{1}H-NMR measurements.

Sample Preparation and ^{1}H-NMR Spectroscopic Analysis OXT (5 mg) was dissolved in 60 μl of D_2O containing 0.75% TSP, 30 μl of 0.2 M phosphate buffer (pH 6.2), and 510 μl of ultrapure water to produce a 600 μl solution for NMR measurements. The sample was introduced into an NMR test tube, and nuclear Overhauser effect spectroscopy (^{1}H-NOESY) spectra were recorded every 7 d at 25 °C using a Varian 600 MHz NMR spectrometer equipped with a coldprobe. Thirty-two free induction decays (FIDs) with 77 K data points per FID were collected using a spectral width of 9615.4 Hz, an acquisition time of 4.00 s, and a total pulse recycle delay of 2.02 s. The

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water resonance was suppressed using by presaturation during the first increment of the NOESY pulse sequence, with irradiation occurring during the 2.0 s relaxation delay and also during the 100 ms mixing time. Prior to Fourier transformation (FT), the FIDs were zero-filled to 128 K and an exponential line broadening factor of 0.5 Hz was applied.[12,13] All peak intensity values (in arbitrary units) were expressed as the means of three separate experiments (n=3). Following each spectrum acquisition, the sample was stored at 4 °C and protected from light.

NMR Data Reduction and Preprocessing All ^{1}H-NMR spectra were phased and baseline corrected by Chenomx NMR Suite 5.0 software, professional edition (Chenomx Inc., Canada). Each ^{1}H-NMR spectrum was subdivided into regions having an equal bin size of 0.04 ppm over a chemical shift range of 0.5—9.0 ppm (excluding the region around the water signal; 4.6—5.0 ppm), and the regions within each bin were integrated. The integrated intensities were then normalized to the total spectra area, and the data was converted from the Chenomx software format into Microsoft Excel format (*. xls). The resultant data sets were then imported into SIMCA-P version 12.0 (Umetrics AB, Umeå, Sweden) for multivariate statistical analysis.

Multivariate Data Analysis PCA was performed to examine the intrinsic variation in the data set.[14,15] The quality of the models was described by R^2x and Q^2 parameters, which indicate the proportion of variance in the data explained by models and goodness of fit. R^2x represents the goodness of fit of the PCA model, and Q^2 reveals the predictability of the PCA model.[16]

Results and Discussion

^{1}H-NMR spectra of OXT obtained at Days 0, 7 and 14 are shown in Fig. 1. While a simple visual inspection suggests that the analysis of a qualitative and quantitative changes might be difficult in three spectra, real spectral differences may be detected if changes can be represented as points in a multidimensional space and examined using PCA. As such, PCA of each OXT spectrum was performed. As a result, distinct differences among the three ^{1}H-NMR spectra were readily detected by both the scores of principal component 1 (PC1) and principal component 2 (PC2), and can be clearly depicted as three separate points as shown in Fig. 2. The PCA modeling revealed R^2x and Q^2 values of 0.80 and 0.49 for PC1, indicating 80% of variance and 49% predictability in the multidimensional space, respectively (Fig. 2). On the other hand, PC2 was explained with a low contribution ratio of 20%. This result suggests a high contribution rate for PC1, indicating that each of the two spectra collected at 7 and 14 d are considerably different from the spectrum collected at Day 0. The differences of the data points on PC1 likely reflect the progress of time-dependent changes of the OXT sample.

The loading plot of all ^{1}H-NMR signals evaluated is shown in Fig. 3. This loading plot reveals the contributions of particular variables (integral regions, in this study) towards either an increase or a decrease in integrated intensities over time. In this case, each variable represents a peak at a particular chemical shift in the ^{1}H-NMR spectral region shown in Fig. 1. From the score and loading plots, the components responsible for increasing or decreasing time-dependent changes can be identified. In addition, the variables at the chemical shifts associated with the largest changes in integrated intensity can be found farther away (to either the left or to the right) from the center of the PC1 coordinate axis.

Eight variables showing typical fluctuations in Fig. 3 were identified, and time-dependent changes of the integrated intensities associated with these variables are shown in Fig. 4. The tendency of the intensity of each of these variables to either increase or decrease is evident, and suggests the formation of new degradation products (Fig. 4A) or the decomposition of the original OXT sample (Fig. 4B).

An increase of the negative value along the PC1 axis in Fig. 3 of four variables (δ 0.82, 2.34, 6.82 and 7.06 ppm) is associated with larger increases in integrated intensity over time (Fig. 4A), and an increase of the positive value along the PC1 axis in Fig. 3 of four variables (δ 0.90, 7.22, 2.06

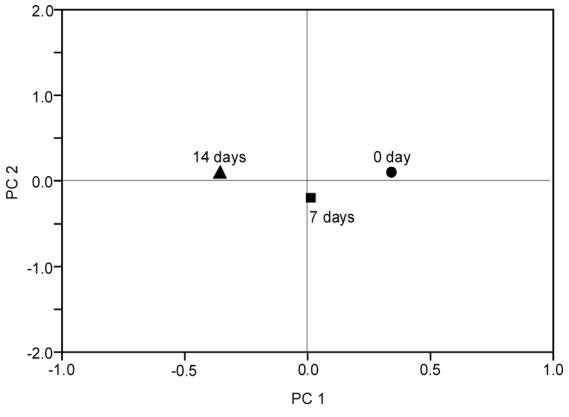

Fig. 2. The PCA Scores Plot Derived from the ^{1}H-NMR Spectra Data of OXT

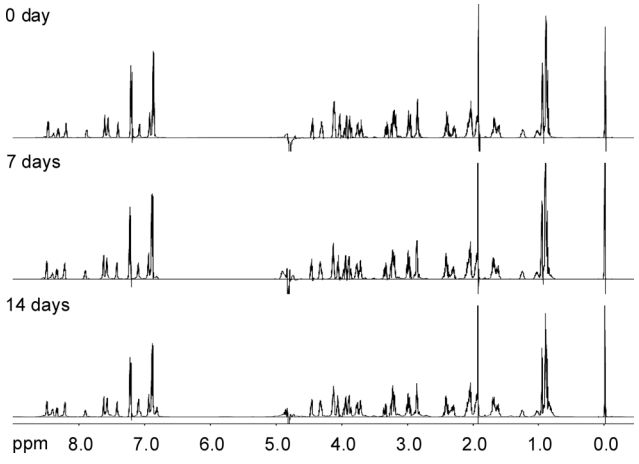

Fig. 1. ^{1}H-NMR Spectra of OXT in 10 mM Phosphate Buffer (pH 6.2) at 25 °C, Collected Every 7 d

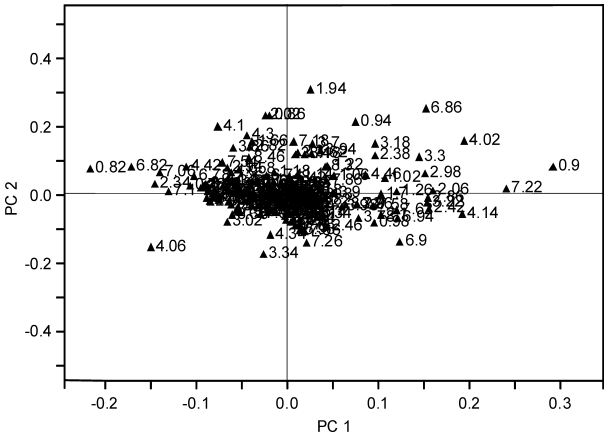

Fig. 3. The PCA Loading Plot Derived from the ^{1}H-NMR Spectra Data of OXT

The variables are shown in chemical shifts, ppm.

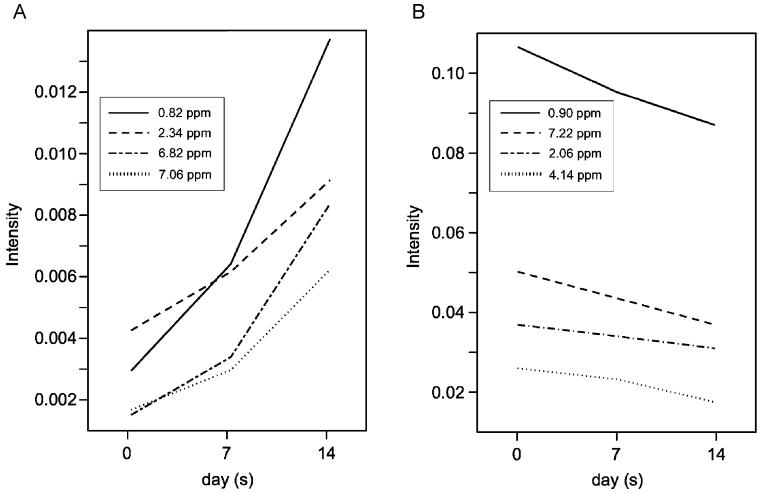

Fig. 4. Time-Dependent Changes in the OXT ^{1}H-NMR Spectrum Showing the Formation of New Decomposition Products (A) and the Decomposition of OXT (B)

and 4.14 ppm) is associated with larger decreases in integrated intensity over time (Fig. 4B). In addition, while the time-dependent increases in intensity for each variable in Fig. 4A are surmised to be due to new peaks from OXT decomposition products of OXT, identifying individual peaks in Fig. 1 associated with these decomposition products is difficult, as these peaks are minor signals. The peaks corresponding to these particular variables were too small to analyze further. On the other hand, peaks for each variable in Fig. 4B could be identified: the variable at 0.90 ppm was attributed to both the δH (δ 0.88) and γ'H (δ 0.89) of Ile and to the δ'H (δ 0.90) of Leu; the variable at 7.22 ppm to the 2′, 6′H (δ 7.22) of Tyr; the variable at 2.06 ppm to the γH (δ 2.05) of Pro and to the βH (δ 2.08) of Gln; and the variable at 4.14 ppm to the αH (δ 4.14) of Gln and to the αH (δ 4.16) of Ile. Therefore, some amino acids for each variable in Fig. 4B are inferred to be associated with OXT decomposition, although proposing a specific decomposition mechanism for OXT is difficult owing to the complexity of protein/peptide systems.

Recently biotechnology-derived drugs for medical treatment are increasingly receiving attention, but many problems associated with the quality, efficacy, and safety of biologics persist. Almost all biologics are designed to mimic human proteins to better predict pharmacological effects, and are thus produced from recombinant or non-recombinant cell-culture expression systems. Preserving the consistency of these complex products during the production is important, as well as reducing or eliminating molecular heterogeneity and higher-order structural aggregates. In addition, since raw materials derived from animals or humans may be used during production,[1)] consideration of possible viral contamination is also crucial. To date, the quality of manufactured biologics is evaluated by measuring biological activity of the biologics, rather than through analysis of physicochemical information. In fact, the product complexity and purity for quality control of biologics are quite difficult to evaluate by current physicochemical methodologies, such as SDS-PAGE, CE, MS, and HPLC.[2—10)]

As a new approach for assessing the quality of biopharmaceuticals, we examined a method to distinguish time-dependent changes of OXT ^{1}H-NMR spectra by introducing PCA of NMR signals. We initially found that simple visual inspection was insufficient to distinguish whether the three ^{1}H-NMR spectra of OXT, gathered every 7 d for 14 d, showed time-dependent changes. However, the PCA scores plot of these same spectra clearly revealed time-dependent changes. In addition, peak changes associated with both new decomposition products and the decomposition of OXT were also observed by the PCA loading plot. Therefore, a combination of ^{1}H-NMR and PCA techniques can provide a molecular fingerprint capable of precisely identifying a protein/peptide biologic, and can represent a powerful new approach for assessing the quality of protein/peptide biologic drugs. The study concerning the feasibility and the limitation of this method in terms of the molecular size is currently under way.

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