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An NMR-Based Similarity Metric for Higher Order Structure Quality Assessment Among U.S. Marketed Insulin Therapeutics

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ABSTRACT

Protein or peptide higher order structure (HOS) is a quality attribute that could affect therapeutic efficacy and safety. Where appropriate, the HOS similarity between a proposed follow-on product and the reference listed drug should be demonstrated during regulatory assessment. Establishing quantitative HOS similarity for 2 drug substances, manufactured by different processes, has been challenging. Herein, HOS differences among U.S. marketed insulin drug products (DPs) were quantified using nuclear magnetic resonance spectra and principal component analysis (PCA). Then, the unitless Mahalanobis distance (D_M) in PCA space was calculated between insulin analog reference listed drugs and their recently approved follow-on products, and all D_M values were 3.29 or less. By contrast, a larger D_M value of 20.5 was obtained between the 2 insulin human DPs independently approved. However, upon mass-balanced and reversible dialysis of the 2 insulin human DPs against the same buffers, the D_M value was reduced to 1.19 or less. Thus, the observed range of nuclear magnetic resonance-PCA–derived D_M values can be used as a robust and sensitive measure of HOS similarity. Overall, the D_M values of 3.3 for DP and 1.2 for drug substances using insulin therapeutics represented realistic and achievable similarity metrics for developing generic or biosimilar drugs, quality assurance, or control.

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Introduction

The number of abbreviated new drug applications referencing peptide and protein therapeutics has grown significantly.[1,2] Typical abbreviated drug application pathways in the United States are generics following 505(j), follow-on new drugs following 505(b)(2), and biosimilars following 351(k). The active pharmaceutical ingredient (API) of these therapeutics, if proteins, must have a correctly folded higher order structure (HOS) to function *in vivo* and effectively deliver the therapeutic dose. Peptides as short as 12 amino acid residues have been shown to form sufficiently stable α-helical structures that could potentially influence peptide activity.[3] In addition to protein secondary and tertiary structure, the HOS can include protein oligomeric structure such as quaternary, quinary, or oligomeric structures.[4,5] The HOS may also be impacted by exchange kinetics among different structural forms in equilibrium.[6] The HOS could present safety risks such as an immune response, for example, if oligomerization at high concentrations becomes irreversible aggregation.[7] Thus, protein or peptide HOS is a quality

Abbreviations used: CQA, critical quality attribute; D_M, Mahalanobis distances; DP, drug product; DS, drug substance; FID, free induction decay; HOS, higher order structure; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser effect spectroscopy; PBS, phosphate buffer saline; PCA, principal component analysis; RLD, reference listed drug; TMSP, trimethylsilyl propanoic acid.

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attribute that may be critical for drug efficacy or safety. For any abbreviated new drug applications (e.g., follow-on products), the HOS quality attribute needs to be demonstrated to be equivalent between the proposed drug and the reference innovator products, usually the reference listed drug (RLD). High-resolution analytical methods used for HOS assessment are needed to capture the complex multiattribute (primary, secondary, tertiary, quaternary, and quinary structures and the exchange kinetics among different structural forms) nature of the API HOS.[8-10]

Solution state protein nuclear magnetic resonance (NMR) spectroscopy is sensitive to changes in chemical composition and HOS through characteristic peak patterns. For example, the NMR peak position or chemical shift depends on amino acid type and H-bond stabilized secondary and tertiary structures[11]; and peak linewidth or spin relaxation rates depend on quaternary, quinary, oligomeric structures and exchange kinetics among equilibrated structural forms.[12] In recent years, one- or two-dimensional (1D or 2D) NMR methods have been used for protein HOS profiling.[4,13-22] These studies support the use of NMR as a robust, reproducible, and sensitive method in identifying HOS changes.[18] Importantly, for HOS assessment, principal component analysis (PCA) on NMR spectral data has been increasingly used as a chemometric approach for spectral comparison.[4,15,23-25] Most of the published studies were performed on drug substance (DS) extracted from drug products (DPs),[4,15,17] or DS available during proprietary drug development.[13,19] However, a limitation of many of these studies was the qualitative data comparison used, which did not yield a numerical measurement of similarity or difference between 2 drug products. A quantitative numerical approach may be beneficial for setting a specification for similarity between comparators for quality assurance, control, and surveillance purposes. However, a practical similarity metric for protein or peptide HOS difference between RLD and the approved follow-on product or between batches among the same product line has not been established.

Several hurdles exist for obtaining realistic HOS similarity metrics. First, marketed biosimilar protein or generic peptide drugs in the United States have not been widely available. For example, the first U.S. protein biosimilar product filgrastim was approved in 2015. Second, formulation differences allowed in biosimilar or generic products of the same drug substance could cause differences in protein or peptide HOS through subtle pH or ionic strength variations. Therefore, separating buffer component–induced HOS changes from drug substance manufacturing–related protein HOS differences may be difficult. Third, it has not been clearly established on which similarity metrics derived from NMR spectra can be used universally without subjective analyst bias or metrics calculated in such a way that lead to a loss in the discriminating power of the test.

To partially address this knowledge gap, recently, we evaluated the 1D ^{1}H and 2D ^{1}H-^{13}C NMR spectral differences between 2 originator DPs of insulin human. Although 2D spectra had much better capability in resolving peaks of excipients and impurities, and differentiating brand-to-brand differences, 1D NMR followed by PCA and Mahalanobis distance (D_M) was sensitive enough and quick for interbrand comparison.[25] In that study, a comparison of the D_M metrics applied between a RLD and an approved follow-on product was not performed.

In this manuscript, U.S. marketed insulin drug products were studied to extend the application of quantitative HOS metrics using 1D NMR, reversible dialysis, PCA, and Mahalanobis distance (D_M) calculation protocols. Insulin therapeutics can have different analogues with modified amino acid sequences to achieve varied pharmacokinetic profiles[26] (Table 1). The chosen insulins were short-acting insulin human, rapid-acting insulin lispro, and long-acting insulin glargine, representing the insulin amino acid sequences of native, mutant B28-K/B29-P, and mutant A21-G/B30a-R/B30b-R, respectively. The 2 insulin human DPs, HumulinR® and NovolinR®, were approved independently as new drugs in 1982 and 1991, respectively. The insulin lispro RLD Humalog® was approved in 1996 and its follow-on drug Admelog® was approved in 2017. The insulin glargine RLD Lantus® was approved in 2000 and its follow-on drug Basaglar® was approved in 2015. The NMR results here demonstrated for the first time a practical similarity metric between both insulin DPs and DSs.

Materials and Methods

Reagents

Sodium sulfate, ethanolamine, and zinc chloride were purchased from Sigma Aldrich (St. Louis, MO). Phosphoric acid, hydrochloric acid (HCl), 10× phosphate-buffered saline (PBS, pH 7.4), 1.0 M sodium acetate buffer (pH 4.0), HPLC grade water, acetonitrile (ACN), and the 2-kDa cut-off Slide-A-Lyzer Dialysis Cassette G2 were purchased from Thermo Fisher Scientific (Waltham, MA). Deuterium oxide (D_2O) and trimethylsilyl propanoic acid (TMSP) were purchased from Cambridge Isotope Laboratories (Boston, MA).

Insulin Drug Products

A total of 14 lots of insulin human (HumulinR® and NovolinR®), 14 lots of insulin lispro (Humalog® and Admelog®), and 14 lots of insulin glargine (Lantus® and Basaglar®) DPs were sourced from the U.S. market (Table S1). All insulin DP formulations had strengths of 100 U/mL and were used directly for NMR measurement. Additional NMR analyses were performed on 14 lots of insulin human drug substances after mass-balanced reversible dialysis (Table S2).

Mass-Balanced Reversible Dialysis

Insulin human drug products HumulinR® and NovolinR® were subjected to a two-step mass-balanced dialysis at 2 different buffer pH values for NMR data collection. For each lot, 1.0 mL of formulation was injected to a G2 Slide-A-Lyzer Dialysis Cassette (Fisher Scientific) and then subjected to the first dialysis against 2.0 L of 25

Table 1
Quantified NMR Spectra Difference Results on Insulin Drug Products (DPs)

Insulin Type	Drug Substance	Drug Product	Approval Type	Year Approved	Buffer	Interbrand D_M
Rapid acting	Insulin lispro	Humalog®	New drug	1996	Intact formulation	3.29
		Admelog®	Follow-on 505(b)(2)	2017		
Long acting	Insulin glargine	Lantus®	New drug	2000		1.58
		Basaglar®	Follow-on 505(b)(2)	2015		
Short acting	Insulin human	HumulinR®	New drug	1982		20.5
		NovolinR®	New drug	1991		

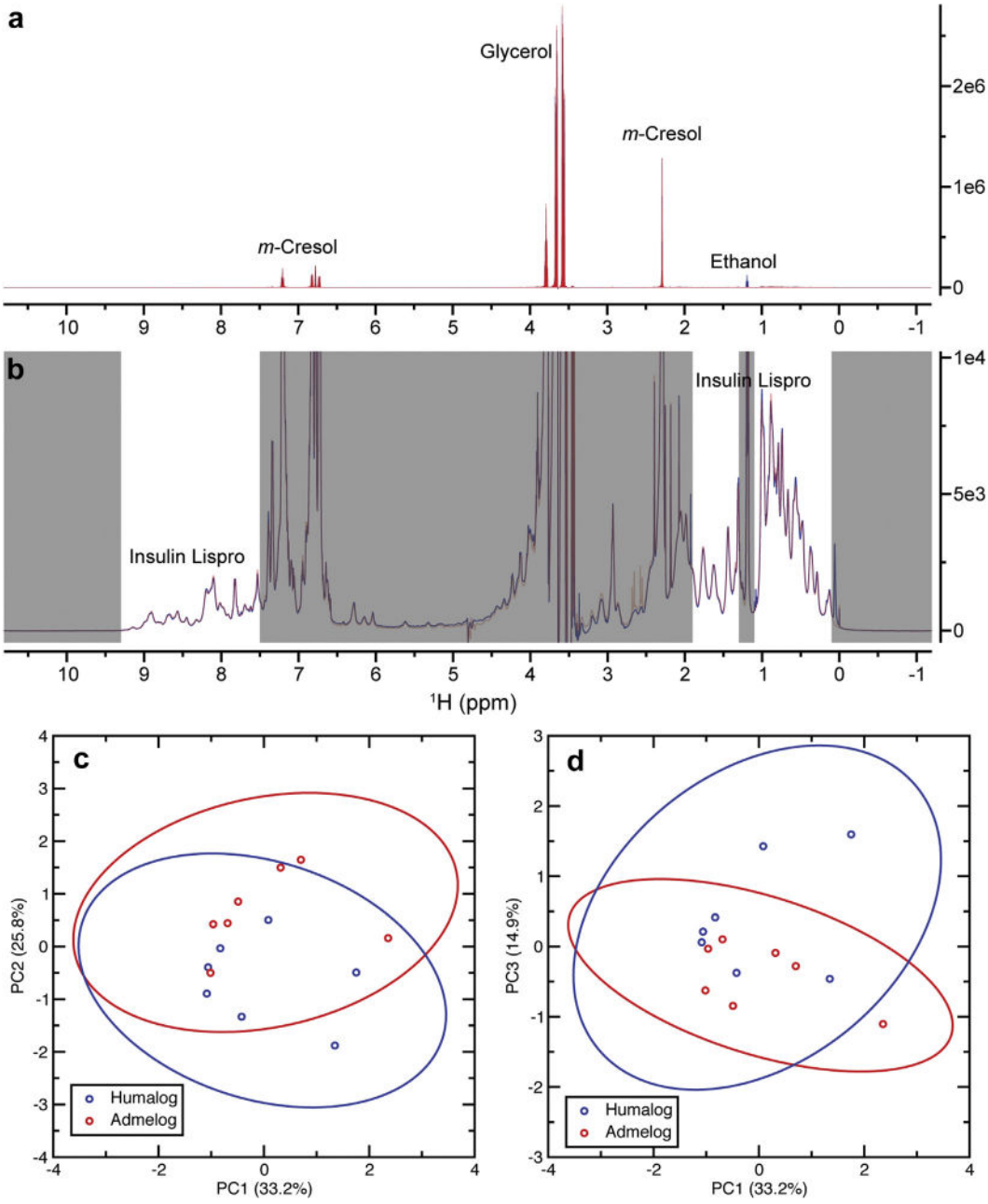

Figure 1. Quantification of spectral differences for insulin lispro drug products (DP), Humalog® (blue) and Admelog® (red). Both the full (a) and the vertically zoomed (b) superimposed 1D ^{1}H NMR spectra of the 2 representative lots of DPs are shown. Assignment for excipients (a) and drug substance (b) are indicated. Blinded spectral regions shown in gray (b) were excluded from the principal component analysis (PCA). The PCA scores are plotted between PC1 and PC2 (c) and between PC1 and PC3 (d). The 90% confidence ellipses are drawn for each brand of insulin lispro.

mM sodium acetate buffer (pH 4.0) with 51 nM zinc chloride at room temperature for 20 h. The second dialysis of the resulting sample inside the same cassette was performed against 2.0 L of 0.5× concentrated PBS (pH 7.4) with 51 nM of zinc chloride at room temperature for 20 h.

HPLC Assay

One milliliter (1.0 mL) of insulin human DP samples taken before and after dialysis were assayed using HPLC. The HPLC method was adapted from the European Pharmacopoeia method for the analysis of insulin human and insulin-related impurities (Table S3).[27] Online separation was conducted on a Waters Acquity H-class UPLC system (Milford, MA) using an Agilent Technologies (Wilmington, DE) Zorbax Eclips XDB C-18 column (5 μm, 4.6 × 150 mm), and detected using an Acquity UPLC PDA Detector (Milford, MA). Separation mobile phase A was 200 mM sodium sulfate buffer containing 56 mM phosphoric acid (pH 2.3) (adjusted to pH 2.3 with ethanolamine, if necessary). Mobile phase B was a mixture of 55% of mobile phase A and 45% acetonitrile. The LC flow rate was 1.0 mL/min, and the column temperature was maintained at 40°C. Insulin drug substance was eluted with an optimized isocratic elution at 55.6% phase B for 35 min. UV data was collected at the absorption wavelength of

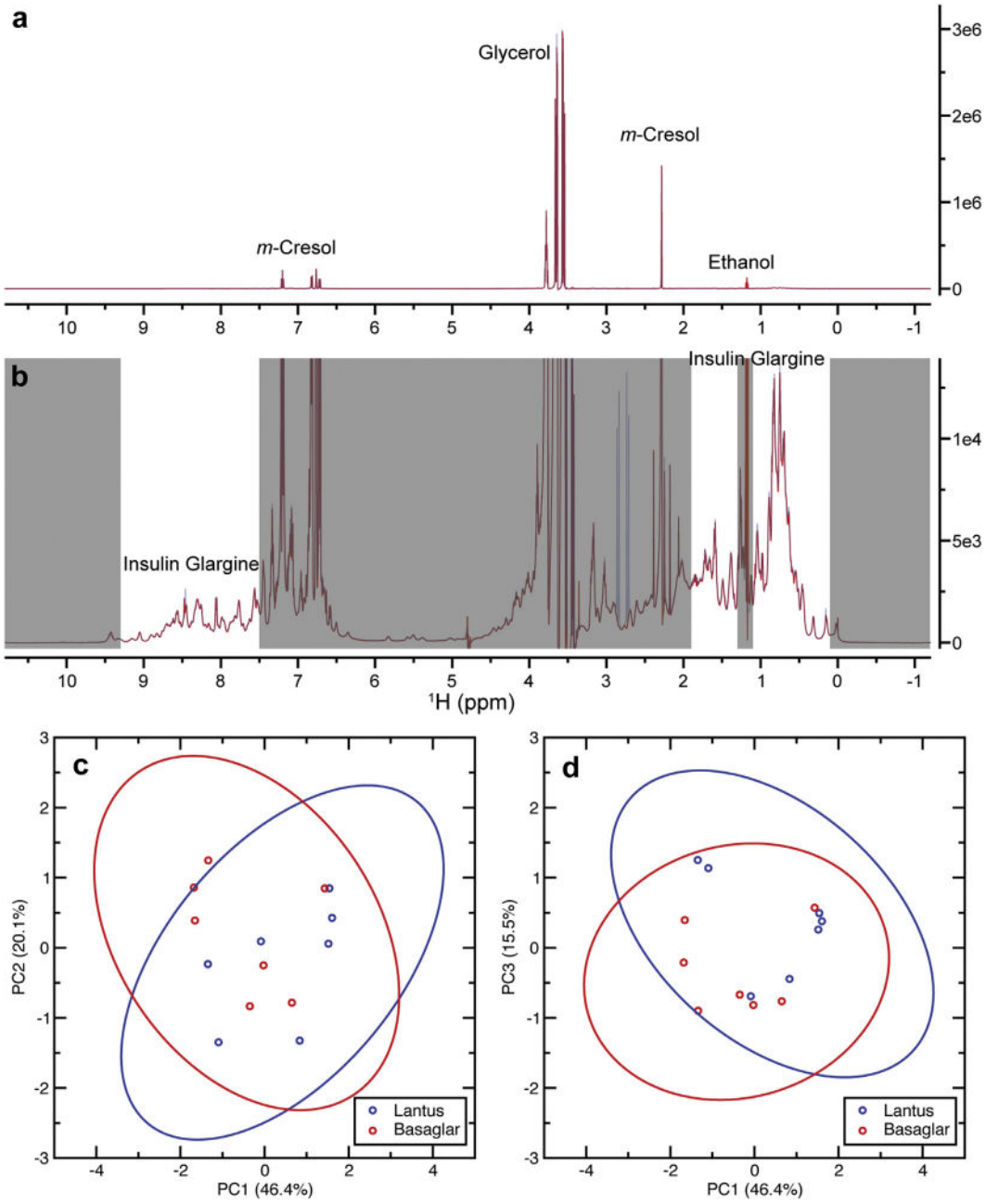

Figure 2. Quantification of spectral differences for insulin glargine drug products (DPs) Lantus® (blue) and Basaglar® (red). Both the full (a) and the vertically zoomed (b) superimposed 1D ^{1}H NMR spectra of the 2 representative lots of DPs are shown. Assignment for excipients (a) and drug substance (b) is indicated. Blinded spectral regions shown in gray (b) were excluded from the principal component analysis (PCA). The PCA scores are plotted between PC1 and PC2 (c) and between PC1 and PC3 (d). The 90% confidence ellipses are drawn for each brand of insulin glargine.

214 nm. Insulin human DP quantity was measured based on detected insulin DS HPLC peak area in reference to that of USP insulin human reference standard.

NMR Samples

For all intact formulations, NMR samples were prepared by directly mixing 500 μL of drug product formulation with 30 μL of deuterium oxide (D_2O) solution which contained 0.002% of TMSP, then transferring to a Wilmad 535-PP-7-5 5-mm NMR precision tube (Wilmad-LabGlass). For dialyzed samples, NMR samples were prepared similarly by aliquoting 500 μL of the insulin human sample from the dialysis cassette after each individual dialysis step was complete.

NMR Spectroscopy

All NMR spectra were acquired on a Bruker Avance III HD 600 MHz spectrometer equipped with a 5-mm TXI triple-axis gradient room temperature probe. The experimental probe temperature was maintained at 25°C. Chemical shifts were internally referenced to TMSP.[28] The 1D ^{1}H NMR data were collected using a 1D NOESY pulse sequence with 3919 Watergate for water suppression as previously reported.[4] The ^{1}H carrier was placed on water resonance

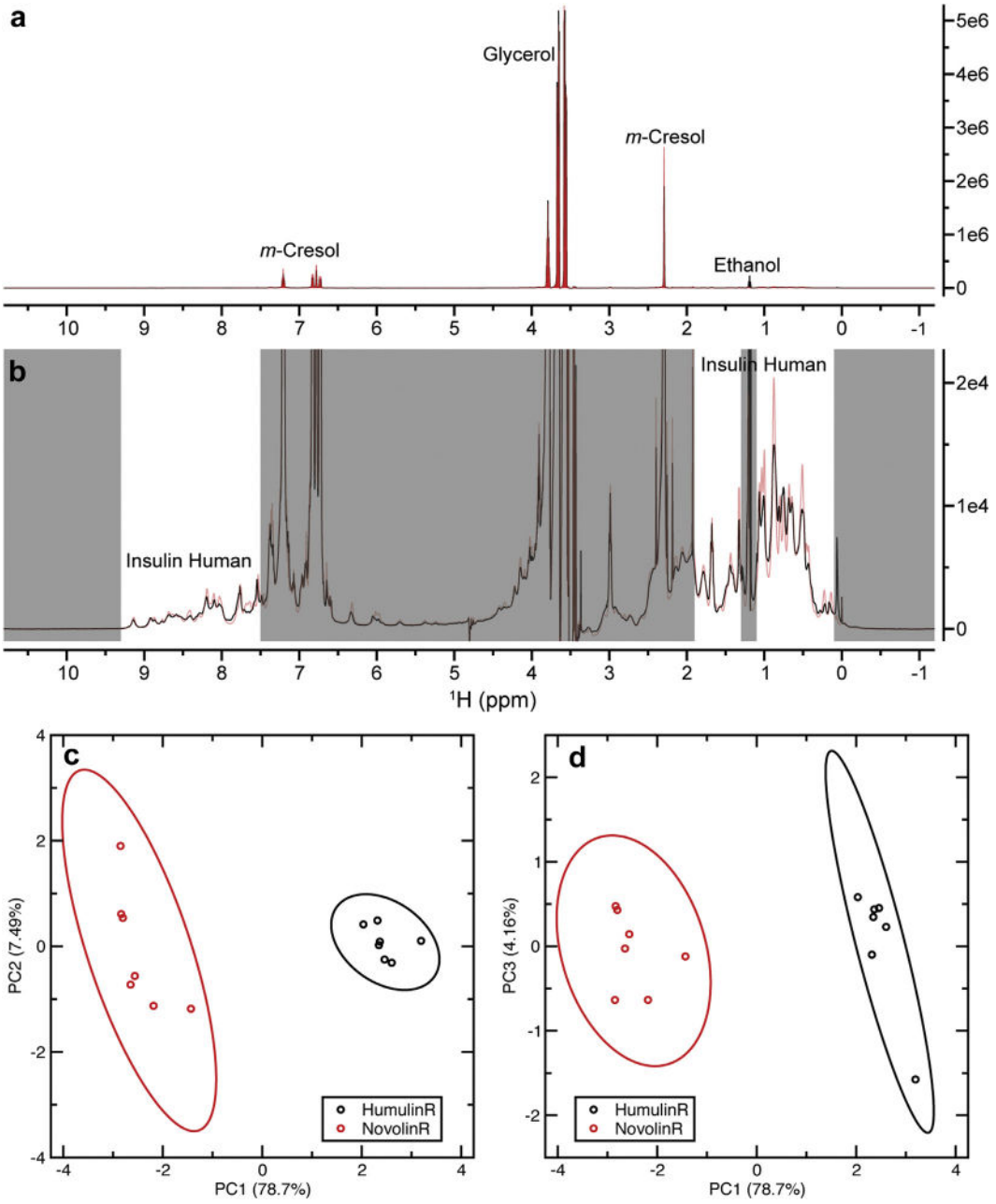

Figure 3. Quantification of spectral differences for insulin human drug products (DPs), HumulinR® (black) and NovolinR® (red). Both the full (a) and the vertically zoomed (b) superimposed 1D ^{1}H NMR spectra of the 2 representative lots of DPs are shown. Assignment for excipients (a) and drug substance (b) is indicated. Blinded spectral regions shown in gray (b) were excluded from the principal component analysis (PCA). The PCA scores are plotted between PC1 and PC2 (c) and between PC1 and PC3 (d). The 90% confidence ellipses are drawn for each brand of insulin human.

at 4.696 ppm. The NOE mixing time was 50 microseconds, and the recycle delay was 2.0 s. The acquisition time was 0.5 s with 7210 complex data points collected in each free induction decay. The spectra width was 12 ppm, and a total of 496 scans were averaged. The NMR experiment time for each 1D spectrum collection was 21.5 min. The raw free induction decays were apodized with a 90° sine square function, scaled half for the first point, and zero filled to spectral size of 8192 points before Fourier transformation. Phase correction with zero order was applied. The resulting line-widths of TMSP peak were all between 2.5 and 3.0 Hz despite some broadening from overlapping insulin peaks at the bottom of the TMSP peaks. All data were processed using MestReNova 12.0.4 software (Mestrelab Research).

Principal Component Analysis

MestReNova 12.0.4 software was used for data processing and PCA. PCA was performed on every 1D ^{1}H NMR spectra groups of the insulin lispro DPs, insulin glargine DPs, insulin human DPs, and insulin human DS after the dialysis. Resonance peaks for the excipients, buffer components, TMSP, and water, corresponding to the regions of <0.1 ppm, 1.10-1.30 ppm, 1.90-7.50 ppm, and >9.30 ppm, were excluded from the PCA analysis. The rest of the regions in the 1D ^{1}H NMR spectra were binned at 0.02 ppm resolution, resulting in a total of 170 bins with summed spectral intensities within each bin. The summed intensities were subject to integrity check, sum normalization, and Pareto scaling before PCA.

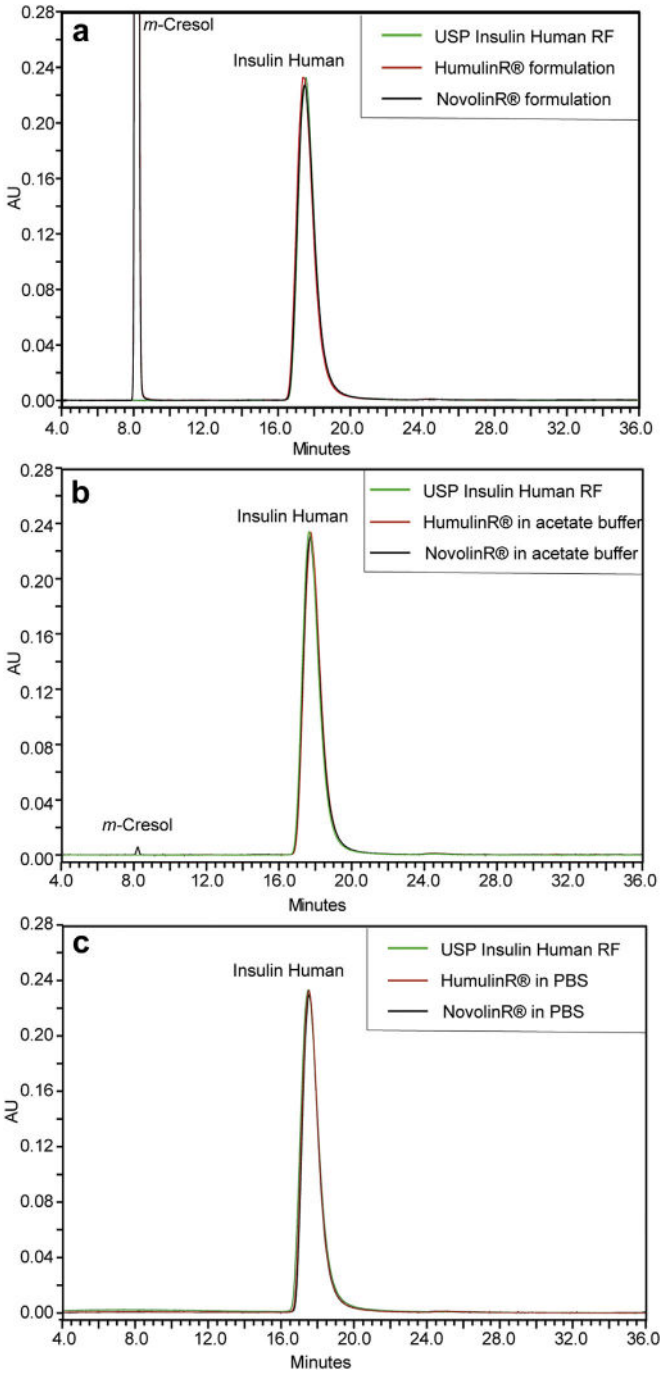

Figure 4. Overlay of HPLC-UV traces (214 nm) of USP insulin human reference standard with (a) HumulinR® DP and NovolinR® DP, (b) HumulinR® DS and NovolinR® DS in 25 mM sodium acetate buffer at pH 4.0, and (c) HumulinR® DS and NovolinR® DS in 0.5× PBS buffer at pH 7.4.

Calculation of Mahalanobis Distances (D_M)

The Mahalanobis distances (D_M) between any 2 insulin brands, for example, A and B, were calculated similarly as before using PCA scores,[25] except that previously the square of the distance (D_M^2) were used to increase the differentiability comparison among methods. Briefly, for each brand, the PC scores from all the lots were tabulated as a sample matrix $A_{m\times n}$, with m representing the number of lots and n representing the number of PC components used toward D_M calculation. In the present study, m was 7 and n was 3. The mean vector $\overline{A}_{1\times n}$ and covariance matrix $S_{An\times n}$ of the sample matrix $A_{m\times n}$ can be calculated. To compare brand A with brand B, the covariance matrices of the 2 need to be averaged per Equation 1 before calculating D_M using Equation 2. The calculations were performed using MATLAB 9.0 (The MathWorks Inc.) and the code can be found in Supplementary information.

$$S = (S_A + S_B)/2 \tag{1}$$

$$D_M = \sqrt{(\overline{A} - \overline{B})S^{-1}(\overline{A} - \overline{B})'} \tag{2}$$

Results

In this study, 1D ^{1}H NMR experiments were performed on multiple drug product lots of Humalog®, Admelog®, Lantus®, Basaglar®, HumulinR®, and NovolinR® (Table S1). In addition, 1D NMR data were collected on the mass-balanced dialyzed insulin human drug substance in 25 mM sodium acetate buffer (pH 4.0) and in 0.5x PBS (pH 7.4), respectively (Table S2).

Quantified Spectral Similarity Between Insulin Drug Products

The 1D ^{1}H NMR spectra were first collected on intact insulin drug products including APIs of insulin lispro (Fig. 1), insulin glargine (Fig. 2), and insulin human (Fig. 3). In the spectra of the 6 drug products, Humalog®, Admelog®, Lantus®, Basaglar®, HumulinR® and NovolinR®, strong peaks from the excipients glycerol, *m*-cresol, and the residual solvent ethanol (Figs. 1a, 2a and 3a) dominated the observed spectral signals because of their high concentration relative to the API insulins. On vertically expanding the spectral scale by 2-3 orders, insulin peaks could be visualized in the regions of 0-4.5 ppm and 5-9 ppm associated with the aliphatic and amide/aromatic proton resonances in the peptides, respectively (Figs. 1b, 2b and 3b). Visually, the spectrum of Admelog®, the follow-on insulin lispro product, showed a comparable spectral pattern to the RLD Humalog® (i.e., similar insulin peak chemical shift and line-width [Fig. 1b]). Similarly, the spectrum of Basaglar®, the follow-on insulin glargine product, showed a comparable spectral pattern to the RLD Lantus® (Fig. 2b). By contrast, the 2 insulin human spectra, HumulinR® and NovolinR®, were different from each other with broader and sharper peaks observed in the HumulinR® and the NovolinR® spectra, respectively (Fig. 3b).[25] Peak line broadening or enhanced spin relaxation could be due to either more insulin oligomerization or intermediate exchange kinetics (i.e., on the micro to millisecond timescale) among different structural forms. Herein the root causes of peak broadening in HumulinR® were not evaluated; however, previous results from diffusion ordered spectroscopy and orthogonal methods like dynamic light scattering suggested insulin oligomer distributions in HumulinR® were similar to or slightly broader than NovolinR®[6]; therefore, intermediate exchange kinetics among different structural forms might be the reason for line broadening in HumulinR®.

Though visual comparison of spectral patterns was informative for determination of HOS equivalence, a quantitative determination was more desirable for regulatory purposes. To quantify the difference in insulin lispro HOS between the RLD Humalog® and the follow-on brand Admelog®, the NMR spectra of 14 lots of insulin lispro from both brands, excluding the spectral regions with peaks of excipients and water (Fig. 1b), were arrayed for chemometric analysis, that is, PCA. The resulting PC1-3 scores were plotted, and

Table 2
Quantified NMR Spectra Difference Results on Insulin Human Drug Substance (DS)

Drug Product	Insulin Human Assay (Unit)	Buffer	Interbrand D_M
HumulinR®	100.5	Sodium acetate (25 mM, pH 4.0)	0.818
NovolinR®	99.7		
HumulinR®	99.9	0.5× PBS (pH 7.4)	1.19
NovolinR®	98.6		

90% confidence interval ellipses were drawn (Figs. 1c and 1d). The first three PC components accounted for 73.9% of spectral variations. The follow-on brand Admelog® spectra had a highly overlapped distribution in PC1-3 spaces with the RLD Humalog® distribution (Figs. 1c and 1d). The Mahalanobis distance (D_M) between the 2 brands of insulin lispro was calculated to be 3.29 when all PC1-3 scores were included in the calculation (Table 1).

The same PCA approach was applied to compare the follow-on brand Basaglar® with insulin glargine RLD Lantus® (Figs. 2c and 2d). The first three PC components accounted for 82.0% of spectral variations. The PCA scores of nearly all drug lots fell into the 90% confidence interval ellipses of both brands, suggesting the interbrand difference was indistinguishable from interlot difference within each brand. The resulting Mahalanobis distance (D_M) was 1.58 (Table 1).

The same procedure was applied to compare the 2 insulin human drug products, HumulinR® and NovolinR®, both of which were innovator drugs (Table 1). For insulin human, the first three PC components accounted for 90.3% of spectral variations. The drug lots from the 2 brands were well separated from each other in PCA space (Figs. 3c and 3d). The calculated D_M value from these data was 20.5 when all PC1-3 scores were included (Table 1). The much larger D_M value suggested a larger HOS difference between insulin humans (HumulinR® and NovolinR®). Because the 2 products were independently approved as new drugs in 1982 and 1991, the insulin expression organism, production, purification, and formulation differences could reasonably result in a larger HOS difference although the API should be the same.

Taken together, the marketed follow-on insulin analogue drugs can have a similarity score as low as 1.58 or 3.29 in Mahalanobis distance (D_M), as evidenced by the approved follow-on drug products of insulin glargine and lispro, respectively. Therefore, the D_M value of 3.3 can be suggested as a realistic and achievable threshold of similarity metrics when comparing the HOS of protein or peptide between 2 drug products or batches in formulation using NMR and PCA.

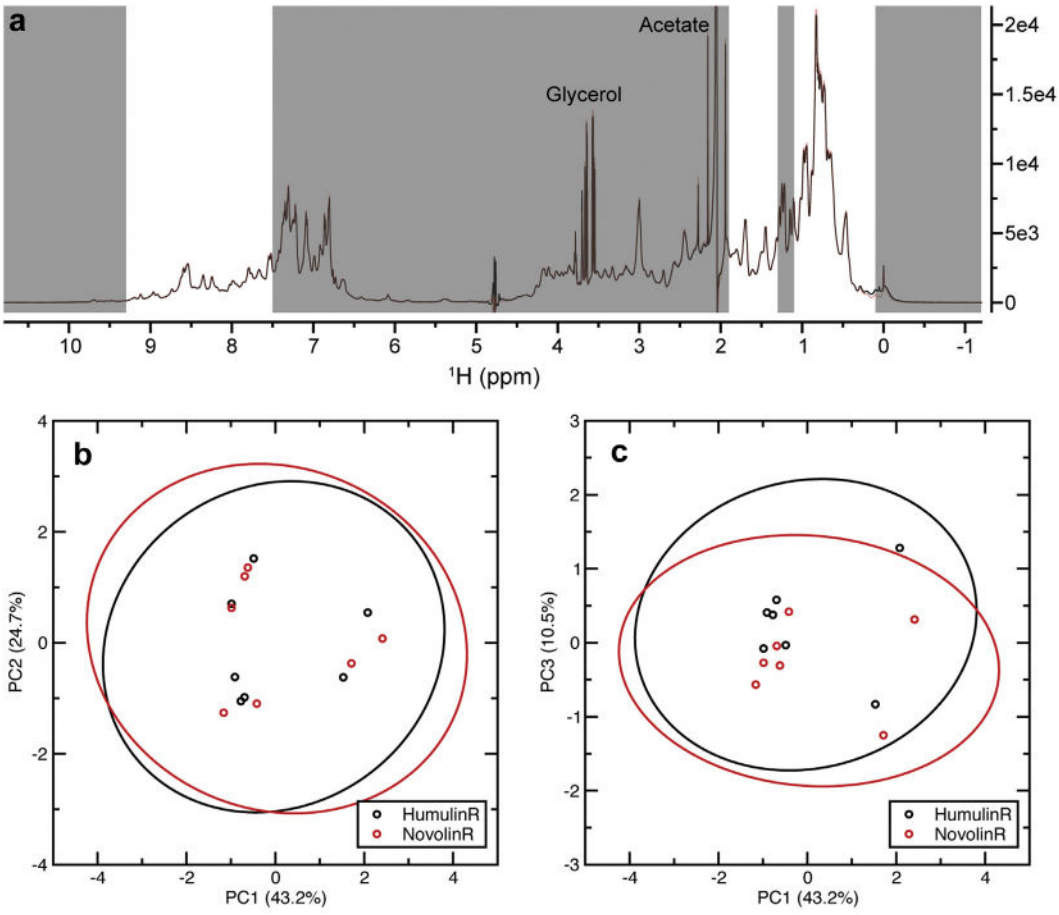

Figure 5. Quantification of spectral difference for insulin human drug substances (DSs) from HumulinR® (black) and NovolinR® (red) in pH 4 buffer. The superimposed 1D ^{1}H NMR spectra of the 2 representative lots of DSs are shown (a). Blinded spectral regions shown in gray were excluded from the principal component analysis (PCA). The PCA scores are plotted between PC1 and PC2 (b) and between PC1 and PC3 (c). The 90% confidence ellipses are drawn for each brand of insulin human.

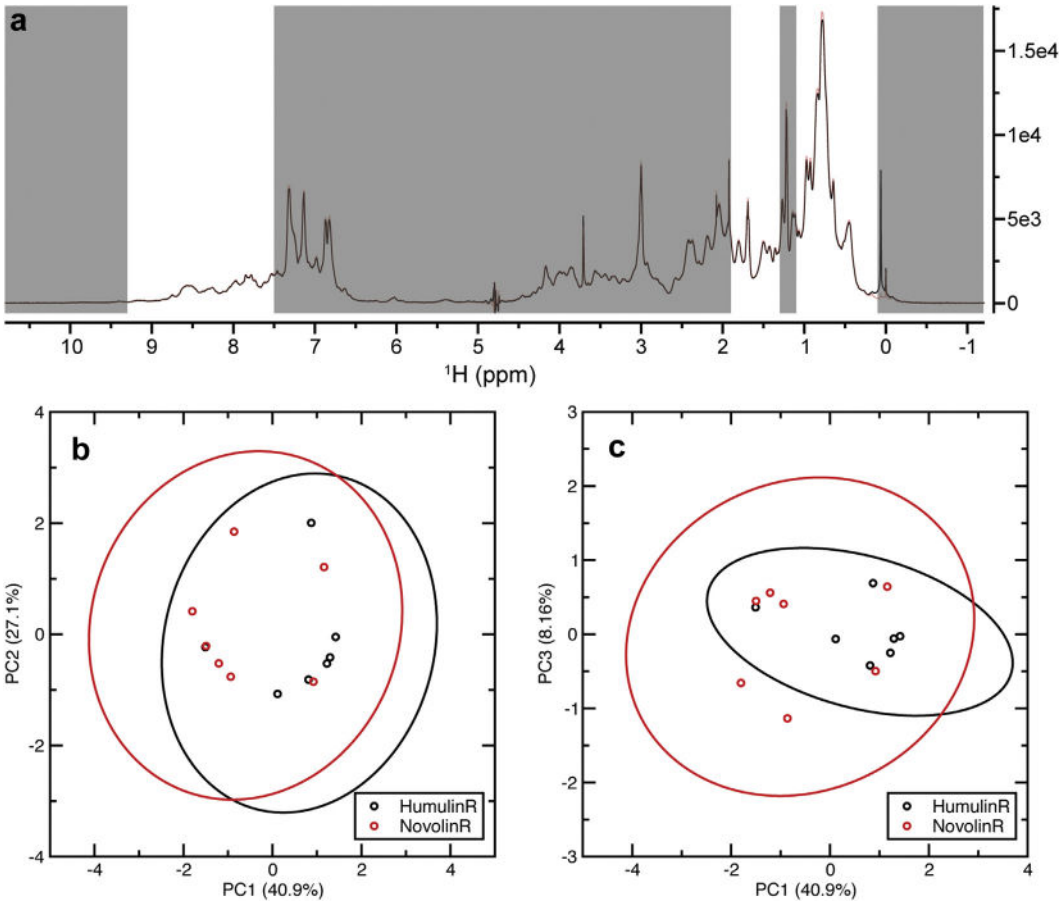

Figure 6. Quantification of spectral difference for insulin human drug substances (DSs) from HumulinR® (black) and NovolinR® (red) in pH 7 buffer. The superimposed 1D ^{1}H NMR spectra of the 2 representative lots of DSs are shown (a). Blinded spectral regions shown in gray were excluded in principal component analysis (PCA). The PCA scores are plotted between PC1 and PC2 (b) and between PC1 and PC3 (c). The 90% confidence ellipses are drawn for each brand of insulin human.

Mass-Balanced Reversible Dialysis of Insulin Human Drug Products

When drug product spectra are not comparable due to differences in manufacture and formulation that affect protein HOS and the potential equilibrium among multimeric forms of insulin, HOS similarity between 2 products may be assessed using the isolated drug substances in identical buffer conditions generated by reversible buffer exchange. For example, NovolinR® and HumulinR® are formulated independently by different manufacturers and would require buffer exchange to make a comparison of the HOS of the insulin drug substances under the same conditions.

Thus, a mass-balanced dialysis was performed on both NovolinR® and HumulinR® to buffer-exchange the insulin human drug substances to the same buffer composition for HOS similarity assessment. Maintenance of the full composition of the drug substance after the mass-balance assay was critical for NMR spectral analysis, as the dialysis protocol should avoid selecting only a subset of insulin human molecules retained through the dialysis procedure. To verify the reversibility of the HOS, the dialysis experiment was designed to be a two-step procedure, first to 25 mM sodium acetate buffer with zinc at low pH 4.0, then to 0.5× PBS with zinc at pH 7.4, which has a pH value closely matched to the insulin human formulation pH value of 7.2-7.4. During dialysis, HPLC assays were applied to monitor insulin loss by measuring the total insulin quantities both before and after dialysis and concomitantly to probe possible formation of any detectable insulin-related degradation products incurred during dialysis. The HPLC results showed that the potencies of intact formulations of HumulinR® and NovolinR® were 101.0 units/mL and 99.6 units/mL, respectively (Fig. 4, Table S4), confirming that both formulations met the USP-required potency range of 95.0%-105.0% of the potency stated on the drug product label. By comparing the total insulin quantities, insulin loss after dialysis was observed to be negligible for testing purposes ($\leq$1.0%) for both HumulinR® and NovolinR® (Table 2), confirming that the dialysis under the designed experimental conditions was a mass-balanced method. In addition, no new insulin peak was observed in the HPLC trace of the dialyzed insulin sample (Fig. 4), suggesting that insulin-related degradants were not produced during the dialysis procedure.

Quantified Spectral Similarity Between Insulin Human Drug Substance

The 1D ^{1}H spectra of the insulin human in the first dialyzed sodium acetate buffer at pH 4.0 were visually indistinguishable for HumulinR® and NovolinR® (Fig. 5a). Each drug substance spectrum had resonances for glycerol and acetate arising from the excipients and dialysis buffer components, respectively. Because of the reduction in excipients after dialysis, the available insulin resonance range for comparison was more than that observed for the spectra obtained on the intact drug products. However, to keep the PCA protocol identical, the same chemical shift regions were retained and binned as that used for the drug product spectra (Fig. 5a). The resulting NMR-derived PC1-3 scores were plotted with a 90% confidence interval ellipse drawn (Figs. 5b and 5c). The 2 ellipses were found to be highly overlapped. The first three PC

components accounted for 78.3% of spectral variations. The D_M value was calculated to be 0.818 using the PC1-3 scores (Table 2).

Next, the insulin human proteins were dialyzed against pH 7.4 PBS buffer. The NMR spectra from pH 7.4 samples were found to be visually different from the spectra at pH 4.0 with broadened lines (Fig. 6a). Most resonances observed were associated with insulin human and much less identifiable excipient or buffer peaks were observed. Nevertheless, the same blind regions were applied to ensure an objective PCA comparison (Fig. 6a). The resulting PC1-3 scores were plotted with a 90% confidence interval ellipse drawn for the 2 brands of insulin DS (Figs. 6b and 6c). The first three PC components accounted for 76.1% of spectral variations. The D_M value was 1.19 when all PC1-3 scores were included in the calculation (Table 2), which was only slightly above the value of spectral comparison at pH 4.0, indicating that a minor change in HOS was observed in pH 7.4 buffer. However, both ellipses were highly overlapped (Figs. 6b and 6c), suggesting the presence of similar HOS for the 2 insulin human drug substances.

Taken together, D_M value of 1.2 can be suggested as a realistic and achievable threshold of similarity metrics when comparing the HOS of the same insulin human drug substance manufactured differently by 2 firms.

Conclusion and Discussion

Among all physicochemical properties, the HOS of a protein or peptide therapeutic can be critical for drug function and stability. NMR spectroscopy, in terms of both NMR peak chemical shift and linewidth, is highly sensitive to HOS changes. From a chemistry, manufacture, and control perspective, the comparison of peptide or protein drug product NMR spectra among different batches and brands may be an important task for drug product development, quality assurance, or quality control.[29] Efforts to quantify NMR spectra differences among different drug brands continue to grow using numeric outcomes from chemical shift mapping and PCA.[4,18,25,30] In a previous study, a chemical shift difference of 8 ppb or less was identified among similar filgrastim drug products obtained from the U.S. and Indian markets.[18] Subsequent work on monoclonal antibody HOS comparison reported even lower thresholds for the same protein across many laboratories.[31] However, most published studies on NMR spectral comparison for peptide or protein HOS of different brands of drugs lack quantification or a numerical similarity score and proposed threshold. Such a numerical score if reproducible and robust could be useful for evaluating the equivalence of generic or biosimilar drugs to the RLD. As previous studies have shown NMR data to be very robust and precise across time, instruments, and laboratories, the D_M metrics such as those reported here have the potential to change how such measurements are reported.

Herein, using U.S. marketed insulin drugs as test articles, similarity scores among 1D ^{1}H NMR spectra of insulin peak regions on both DPs and DSs were calculated. The choice of 1D NOESY ^{1}H NMR pulse sequence program was primarily based on the satisfactory water suppression observed with this pulse sequence at 600 MHz.[4] In practice, any pulse scheme that has suitable water suppression capability could be applied to maximize the API signals for comparison. In performing comparison of insulin HOS, excluding excipients, residual solvent, and process-related impurity peaks was necessary as these signals do not reflect the structure of the drug substance. These signals were identifiable based on chemical shifts and characteristic sharper peaks compared to the insulin signals. Of note, depending on the instrument, the NMR measurement may not be sufficiently sensitive to low levels (~<5%) of protein degradation−related impurities (e.g., asparagine deamidation) or other post translational modifications; therefore, the comparison of insulin peaks mostly represents the major HOS quality attributes.

Similarity scores for the observable HOS relevant signals were expressed in the unitless Mahalanobis distance (D_M) derived from PCA space with an unsupervised chemometric approach. As a result, between the 2 U.S. marketed rapid-acting insulin lispro DPs, the RLD Humalog® and the follow-on Admelog®, which were approved in 1996 and 2017, respectively, a similarity score of D_M of 3.3 was obtained. Between the 2 U.S. marketed long-acting insulin glargine DPs, the RLD Lantus® and the follow-on Basaglar®, which were approved in 2000 and 2015, respectively, a similarity score of D_M of 1.6 was obtained. Thus, a D_M value of 3.3 could be proposed as a realistic and achievable similarity threshold for a generic drug product manufactured by a drug firm with limited chemistry, manufacture, and control knowledge of the RLD product.

In parallel, when only the drug substance between different formulations needs to be compared, the deformulation process should assure the reversibility of protein HOS change without loss of drug substance. The drug substance comparison procedure has been demonstrated here using the drug products HumulinR® and NovolinR®. The 2 insulin human DPs were independently approved without any regulatory tie of RLD and generics or follow-ons, and both contained drug substance insulin human, expressed in different organisms.[32] The two-step dialysis design at different pHs with an HPLC assay assured the reversibility of insulin human HOS with retention of over 98% of drug substance assay. The resulting D_M value was 1.2 or less could be proposed as a second achievable similarity threshold for drug substance similarity between active pharmaceutical ingredients found in drug products manufactured by 2 independent firms.

In this work, the large dynamic range of D_M values from 0.818 to 20.5 (Tables 1 and 2) observed for the insulin data illustrated high sensitivity of NMR spectral differences to protein HOS. One indication that these threshold values may be widely applicable is that in a similar approach (i.e., using 1D NMR, PCA, and D_M calculation) reported by Goodpaster and Kennedy for the evaluation of the separation threshold among spectra in a metabonomic study,[33] a D_M value below 1.4 was indicative of partial to no separation while a value of 7.7 was indicative of total separation. Although the subject of study was completely different from drug quality, the partial-separation threshold in metabolomics expressed in D_M was similar to the current D_M values derived from insulin DS comparison of 1.2. Overall, the D_M for drug product similarity threshold value of 3.3 or drug substance value of 1.2 require additional testing to establish their usefulness over time and with a wider set of products where originator and follow-on/generic/biosimilar comparators are available.

In addition to providing HOS similarity thresholds for drug products or drug substances, the work here is presented using a commonly available NMR spectrometer (i.e., 600-MHz with a room-temperature probe) and simple 1D ^{1}H spectra, as sufficient for HOS quality comparisons. Notably, the chemometric approach is generic and would be equally applicable to any solution or solid-state NMR spectra and even other lower resolution spectroscopy like circular dichroism that compares primarily secondary structure elements. As such, the NMR and PCA approach, in addition to being applicable as a quality assurance tool, could be a routine quality control tool for release testing or surveillance measurements to assure drug product quality over the lifecycle of a drug.

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