



Research paper

Structural meta-analysis of regular human insulin in pharmaceutical formulations



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ABSTRACT

We have studied regular acting, wild-type human insulin at potency of 100 U/mL from four different pharmaceutical products directly from their final finished formulation by the combined use of mass spectrometry (MS), dynamic light scattering (DLS), small-angle X-ray scattering (SAXS), nuclear magnetic resonance (NMR), and single-crystal protein crystallography (PX). All products showed similar oligomeric assembly in solution as judged by DLS and SAXS measurements. The NMR spectra were compatible with well folded proteins, showing close conformational identity for the human insulin in the four products. Crystallographic assays conducted with the final formulated products resulted in all insulin crystals belonging to the R3 space group with two a dimer in the asymmetric unit, both with the B-chain in the T configuration. Meta-analysis of the 24 crystal structures solved from the four distinct insulin products revealed close similarity between them regardless of variables such as biological origin, product batch, country origin of the product, and analytical approach, revealing a low conformational variability for the converging insulin structural ensemble. We propose the use of MS, SAXS, NMR fingerprint, and PX as a precise chemical and structural proof of folding identity of regular insulin in the final, formulated product.

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1. Introduction

The onset of diabetes mellitus (DM) and loss of control of metabolism affects a large part of the world population [1,2]. Large effort has been made for the development of new therapeutics for the control of DM as well as in the search for its cure [3–5,5–11].

However, therapeutic reposition of pancreatic hormones – such as insulin and amylin and their analogs – has not been replaced and still remains an important therapy [12–14] which motivates the worldwide development and revision of process for the

production of insulin, new insulin products, analogs, and delivery systems [3,15–20] in addition to the large portfolio of insulin products and analogs currently available from different brands.

Along with the inherent difficulties of developing and producing a biological products for therapeutic use, characterization of the final product is also challenging and demands a continuous revision process of analytical methods and quality requirements applied for pharmaceutical ingredients, finished and in-process products.

Protein therapeutics are a class of products which have a complex three dimensional structure in solution whose integrity determines the biological activity, clinical efficacy, and safety. Thus, it is highly desirable that products from this class meet well-defined requirements for structural integrity. Proteolytic footprint has long been the method of choice, generating a peptide mapping which indirectly provides evidences for solution structural arrangement [21]. Previous work have shown that slight changes in excipient used in insulin formulation can result in redistribution of

Abbreviations: NMR, nuclear magnetic resonance; SAXS, small-angle X-ray scattering; MALDI-TOF, matrix-assisted laser desorption/ionization – time-of-flight; hINS, human insulin.

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oligomeric forms [22,23]. However, besides its high analytical precision and sensitivity, this approach is not sufficient to provide the exact three-dimensional structure of these biological entities.

Still remains the question whether the assessment of structural information could be satisfactorily performed in the final finished biological product, providing chemical information about the molecular integrity and the detailed structural information about the exact three-dimensional structure of these biological entities in its final formulations. To address this issue, we have conducted an extensive structural and spectroscopic characterization of regular-acting, wild-type human insulin formulations at same potency of 100 U/mL from four distinct brands.

Insulin is a protein hormone used worldwide in the treatment of diabetes. Its active form is comprised of polypeptide chains A and B, linked by three disulfide bonds. Insulin crystals have been investigated for decades [24–26], and the insulin high resolution structure has long been known from varying crystallographic techniques such as single crystals [27] and microcrystalline powder [28] diffraction. NMR has also been used in the structural characterization of insulin in its varying oligomeric and conformational states, such as monomers [29], T₆, T₃R₃ [30] and R₆ hexamer [31] and in comparative studies with diverse mutants [32–35] and also in diverse solution composition [32,34,36]. The choice of the products used in the present study was based on the fact that they are pharmaceutically available for therapeutic use and are produced by four dissimilar methods such as the heterologous recombinant production (in *Escherichia coli*, *Saccharomyces cerevisiae* or *Pichia pastoris*) and by the semi-synthetic approach (by the modification of the aminoacid Thr^{B30} in porcine insulin), also varying in the excipient composition.

2. Experimental

2.1. Chemicals

Human insulin formulations at 100 U/mL were acquired direct from pharmaceutical drug stores and kept under refrigeration at 4 °C until use. We used in this work several insulin batches, as depicted in Table 1. We did not detect aggregates according to the DLS and SAXS measurements (Table 3 and Fig. 1).

We have also used the insulin analogs Asp^{28B} (Aspart insulin, NovoRapid Penfill Aspart Insulin lot # XS62590) [37] and Lys28B Pro29B (LisPro insulin, Humalog Lispro Insulin lot # a696780) [38] for the evaluation of the selectivity of the crystallization conditions (see below). All other reagents were of analytical grade.

2.2. Mass spectra using MALDI-TOF

Insulin formulations were diluted 10 times with acetonitrile/water 50:50 (v/v) containing 0.1% trifluoroacetic acid (TFA) v/v. Alfa-cyano-4-hydroxycinnamic acid (CHCA; 10 mg/mL in CHCN₃, 0.1% TFA in water; 2:1, v/v) was used as the matrix agent in a 1:1 mixture with the insulin solution in CHCN₃. 1 μL of CHCA-insulin mixture was applied to the sample plate and air-dried at room temperature. The MALDI-TOF mass spectra were recorded using an Applied Biosystems Voyager instrument from Proteomics Facility at IBqM-UFRJ, calibrated using external standards at each 2 sample measurements.

2.3. Dynamic light scattering

Particle size distribution of the human insulin formulations was evaluated ($n = 3$) at 25 °C by dynamic light scattering in a DynaPro NanoStar (Wyatt Technology, USA) previously calibrated according to manufacturer. The relative percentage polydispersity (% PD) was

Table 1

Summary of human regular insulin formulation used in this study.

Product/ composition ^a	Company	Batch number	Country of manufacturing ^a	Country of acquisition
Humulin® R Human insulin (rDNA origin; <i>E. coli</i>) 100 units/mL, glycerol 16 mg/mL, metacresol 2.5 mg/mL, endogenous zinc (0.015 mg Zn), pH 7.0–7.8	Eli Lilly	A405936	Mexico	Brazil
		A560347	Mexico	Mexico
		A505073	EUA	Argentina
Novolin® R Human insulin (rDNA origin; <i>S. cerevisiae</i>) 100 U/mL, glycerol 16 mg/mL, metacresol 3 mg/mL, endogenous zinc (0.007 mg), pH 7.4	Novo Nordisk	XS69303	Denmark	Brazil
		TS62987	Denmark	Mexico
		TS62953	Denmark	Argentina
Insunorm® R Human insulin (rDNA origin; <i>P. pastoris</i>) 100 U/mL, endogenous zinc, glycerol, metacresol	Cellofarm	I550005	India	Brazil
Betalin® H Human insulin (semi-synthetic) 100 U/mL, endogenous zinc, glycerol, phenol	Beta Lab	310996-1	Argentina	Argentina

^a From their respective product leaflet and [115]. The amount of each excipients for Insunorm and Betalin was not disclosed by the manufacturer.

evaluated from the number average intensity, representing the percentage of the peak size half width divided by the mean peak size.

2.4. Small angle X-ray scattering

SAXS experiments were carried out at the D11-SAS1 beam line [39] on the LNLS. SAXS data were collected directly from the regular human insulin formulations using a two-dimensional detector (MarCCD; MarResearch, USA) at wavelength 1.488 Å with a sample-detector distance providing a q -range from 0.02 Å⁻¹ to 0.25 Å⁻¹, where q is the modulus of the scattering vector (calculated according to $q = (4\pi/\lambda) \sin \theta$, where λ is the wavelength used and 2θ is the scattering angle). The scattering curves of the protein solutions and buffers were collected in several successive frames of 500 s each to monitor for radiation-induced protein oxidation. The data reduction routine included normalization of the one-dimensional scattered data to the intensity of the transmitted incident beam, correction for the detector response, incident beam intensity, sample absorption, and blank subtraction using scattering from water.

A Guinier analysis of solution scattering [40] was applied to evaluate the monodispersity of each specimen and to determine the radius of gyration (R_g) of Insulin R in solution. The R_g and the scattered intensity, $I(q)$, were inferred, respectively, from the slope and the intercept of the linear fit of $\ln[I(q)]$ versus q^2 in the q -range $q * R_g < 1.3$ [40]. The same parameter was also obtained from the data fit of the merged curve by the indirect Fourier transform program Gnom [41], also evaluating the distance-distribution

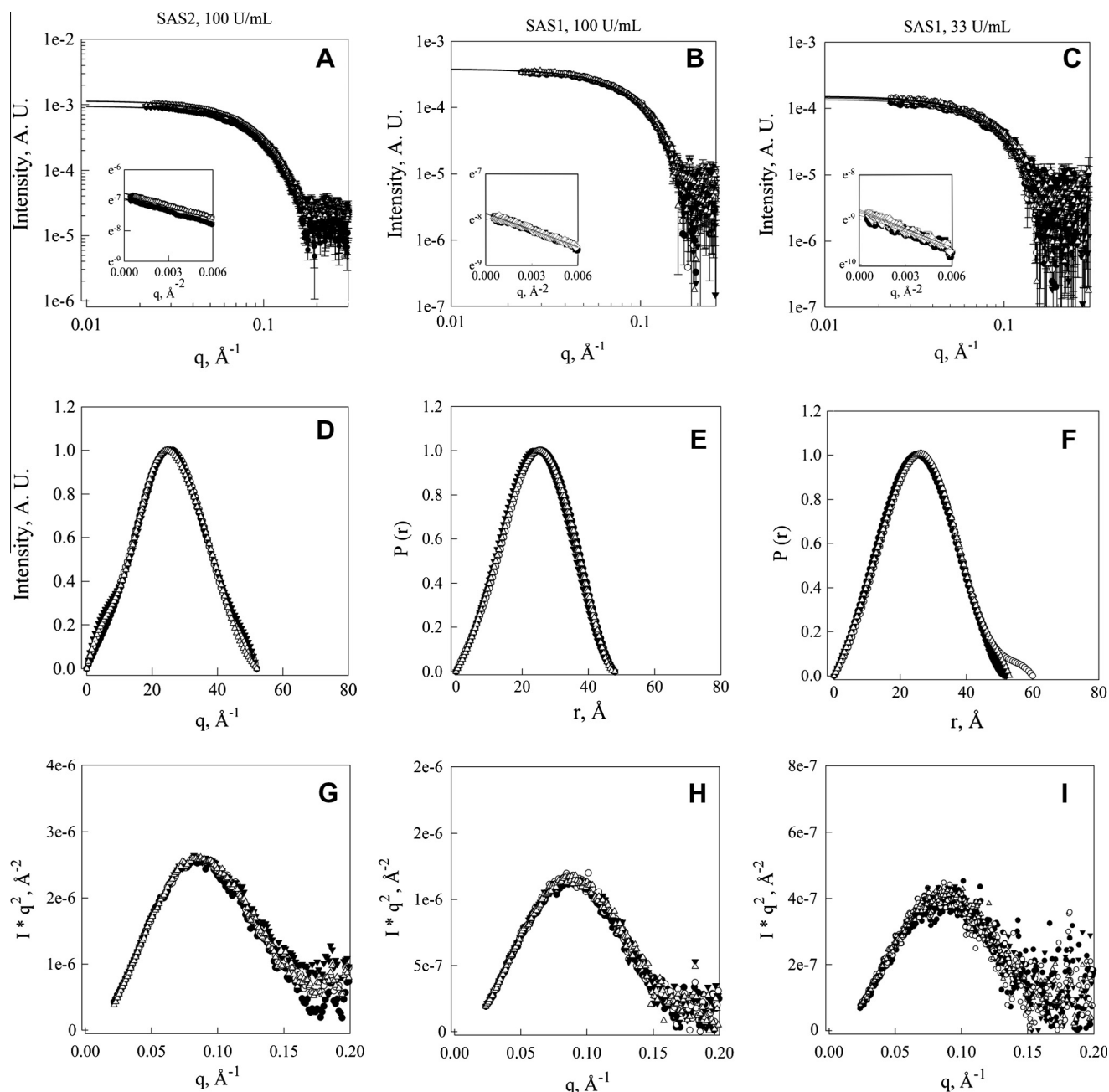


Fig. 1. Small-angle X-ray scattering (SAXS) profile of regular insulin. Scattering profiles of insulin formulations: Humulin R[®] (●), Novolin R[®] (○), Insunorm R[®] (▼), Betalin H[®] (△). SAXS measurements were performed with two different insulin batches for each product at two distinct beamlines, SAS2 (A, D, G) and SAS1 (B, C, E, F, H, I) of insulin formulations at (A, B, D, E, G, H) at 100 U/mL and (C, F, I) 33 U/mL (diluted with type-I water immediately prior measurements). (A, B and C) $I(q)$ scattering curves. *Inset*: Guinier plot. The linearity of the Guinier plot indicates that insulin solutions are monodispersed. Lines correspond to best adjust with Gnom. (D, E and F) pair-distance distribution for insulin formulations. (G, H and I) Kratky plot for insulin formulations. Details in Section 2 and in Table 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

function, $P(r)$, of Insulin R. The maximum dimension, D_{\max} , was estimated from the distance distribution function $P(r)$, with the limiting distance r where $P(r)$ first converges down to zero.

2.5. Protein crystallography

Crystals of the human insulin were obtained by hanging drop at temperature varying from 18 °C to 25 °C as specified (Table S1), by direct mixing 2 μ L of the insulin formulations with 2 μ L of well solution and equilibrating against 500 μ L of well solution. Crystals of approximately 50–100 μ m grew in about 2 days. The crystals were quickly soaked in well solution supplemented with varying

glycerol concentrations and subjected to X-ray diffraction and data collection under nitrogen stream at 100 K (with previous flash-cooling in liquid nitrogen or by direct flash-cooling with the nitrogen stream, as depicted in Table S1) in varying setups, as follow:

- the MX1 synchrotron beamline [42] from the National Synchrotron Light Laboratory, Campinas, Brazil (LNLS-CNPEM), recorded on a MARCCD165;
- the MX2 synchrotron beamline [43] from the National Synchrotron Light Laboratory, Campinas, Brazil (LNLS-CNPEM), with the diffraction intensities recorded using a MarMosaic 225 detector (MAR Research GmbH, Norderstedt, Germany);

Table 4
Small-angle X-ray scattering analysis of insulin products.

	Humulin [®] R	Novolin [®] R	Insunorm [®] R	Betalin [®] H
<i>SAS1 (100 U/mL)</i>				
Rg (Å)				
Guinier	18.8 ($r^2 = 0.971$)	18.9 ($r^2 = 0.976$)	19.0 ($r^2 = 0.980$)	19.0 ($r^2 = 0.978$)
$P(r)$	18.6 ± 0.03	18.5 ± 0.03	18.8 ± 0.04	18.5 ± 0.04
D_{\max} (Å)	48 ± 2	48 ± 2	50 ± 2	48 ± 2
<i>SAS1 (33 U/mL)</i>				
Rg (Å)				
Guinier	19.0 ($r^2 = 0.928$)	20.1 ($r^2 = 0.952$)	19.1 ($r^2 = 0.929$)	20.1 ($r^2 = 0.946$)
$P(r)$	19.2 ± 0.07	20.2 ± 0.07	19.3 ± 0.09	19.7 ± 0.06
D_{\max} (Å)	52 ± 2	60 ± 2	52 ± 2	53 ± 2
<i>SAS2 (100 U/mL)</i>				
Rg (Å)				
Guinier	20.4 ($r^2 = 0.998$)	19.9 ($r^2 = 0.998$)	20.1 ($r^2 = 0.998$)	19.8 ($r^2 = 0.997$)
$P(r)$	19.6 ± 0.03	19.45 ± 0.02	19.63 ± 0.03	19.3 ± 0.03
D_{\max} (Å)	52 ± 2	52 ± 2	52 ± 2	52 ± 2

- a home source, using Cu K α radiation generated by a Rigaku UltraX 18 rotating anode operated at 50 kV and 90 mA, equipped with Osmic confocal Max-Flux optics and recorded on a MAR 345dtb image plate;
- a home source, using Cu K α radiation generated by a NOVA source installed in a SuperNova diffractometer (Agilent) operated at 50 kV and 0.8 mA and recorded on a Titan (Agilent) area detector.

The images were indexed and processed with Mosflm [44] or CrysAlisPro [45] and scaled with Scala [46]. The complex was solved by molecular replacement using MolRep [47] or Phaser [48] and human insulin crystal structure (PDB entry: **3I3Z**) as search model, resulting in a clear solution for a dimer in the asymmetric unit. The structure was further refined using Refmac [49]. Real space refinement was conducted by visual inspection of both the map and model with Coot [50], which was also used for the addition of water molecules and zinc ions. A summary of crystal parameters and data collection and refinement statistics are presented in Table 4. Structural validation of the model performed with PROCHECK [51] showed that all main-chain dihedral angles were in the allowed regions. All residues were found in favorable Ramachandran regions. A detailed report of structure statistics can be found in Table S1. All figures were generated with PyMOL [52]. Global pairwise alignment of the insulin structures was performed using Pro-SMART [53] considering the two monomers in the asymmetric unit as a single structural unit, to achieve the global RMSD corresponding to the superposition of two pairs of monomers. The atomic coordinates have been deposited with the Protein Data Bank and have been assigned the codes as depicted in Table S1.

Table 2
Mass spectrometry analysis of human insulin.

Formulation/brand	Batch number	m/z
Humulin [®] R	A405936	5809.92
	A560347	5808.89
	A505073	5809.07
Novolin [®] R	XS69303	5809.93
	TS62987	5808.94
Insunorm [®] R	I550005	5809.92
Betalin [®] H	310996-1	5809.07

Table 3
Dynamic light scattering analysis of human insulin formulations.

	Hydrodynamic radius (Rh) (Å)	Polydispersity (Pd) (%)
Humulin [®] R	17.0 ± 0.3	3.8 ± 0.9
Novolin [®] R	20.2 ± 0.4	4.5 ± 0.4
Insunorm [®] R	19.6 ± 1.4	4.4 ± 0.3
Betalin [®] H	18.9 ± 2.5	3.3 ± 1.4

2.6. Nuclear magnetic resonance

The NMR measurements were performed with the following human insulin products and their respective batch number: Humulin[®] R (Eli Lilly; Brazil) lot #A562606; Novolin[®] R (Novo Nordisk, Brazil) lot #TS62953; Insunorm[®] R (Cellofarm, Brazil) lot #I550005; Betalin[®] H Corriente Semisintetica lot #44713. Samples was prepared by adding D₂O (~10% v/v final concentration) directly to each insulin formulation. NMR experiments were performed on a 600 MHz Bruker Avance III spectrometer equipped with a cryogenic probe (from the CNRMN-UFRJ) using pulse programs implemented in Bruker Top Spin pack and on a Varian 500 MHz INOVA also equipped with a cryogenic probe (from the LNBio-CNPEN) using pulse programs implemented in VNMRJ v 2.1. Temperature of 25 °C was used for all experiments. For 2D ¹³C-HSQC spectra (for Bruker pulse sequence hsqcetgp, avance-version 07/04/04 or for Varian pulse sequence gChsqc), we used natural abundant ¹³C signal of samples and 1024 transients in ¹H dimension and 128 transients in ¹³C dimension. Data from Bruker were processed with Bruker Top Spin pack or data from Varian were processed with nmrPipe suite [54] and analyzed with Computer Aided Resonance Assignment (CARA [55]).

3. Results

3.1. Molecular weight determination by mass spectrometry

We have conducted an initial characterization of the four regular insulin products by mass spectrometry. The MALDI-TOF analysis showed a peak with m/z of 5809 for all insulin products (Table 2). This result is in agreement with the expected molecular mass for wild-type, human insulin and thus confirms the chemical identity and integrity of the insulin product in the formulation tested, regardless of the manufacturing process.

3.2. Evaluation of the oligomeric organization by dynamic light scattering

We have evaluated the oligomeric properties of the regular insulin products in the pharmaceutical formulation by dynamic light scattering (DLS). We have observed similar particle behavior in solution for the insulin from the insulin formulation from four distinct products, as evidenced by the close hydrodynamic radius within error, ranging from 18 to 20 Å and also close value of polydispersity within error, ranging from 3.3% to 4.5% (Table 3).

3.3. Evaluation of the structural organization by small-angle X-ray scattering

In order to gain more insight on the structural organization of the insulin products in their pharmaceutical formulation, we have performed small-angle X-ray scattering (SAXS) measurements, at two different SAXS beamlines using two distinct insulin batches from each product. In particular, SAXS has long being used to identify the quaternary organization of proteins, including insulin and insulinomimetic compounds at varying compositions [56–58].

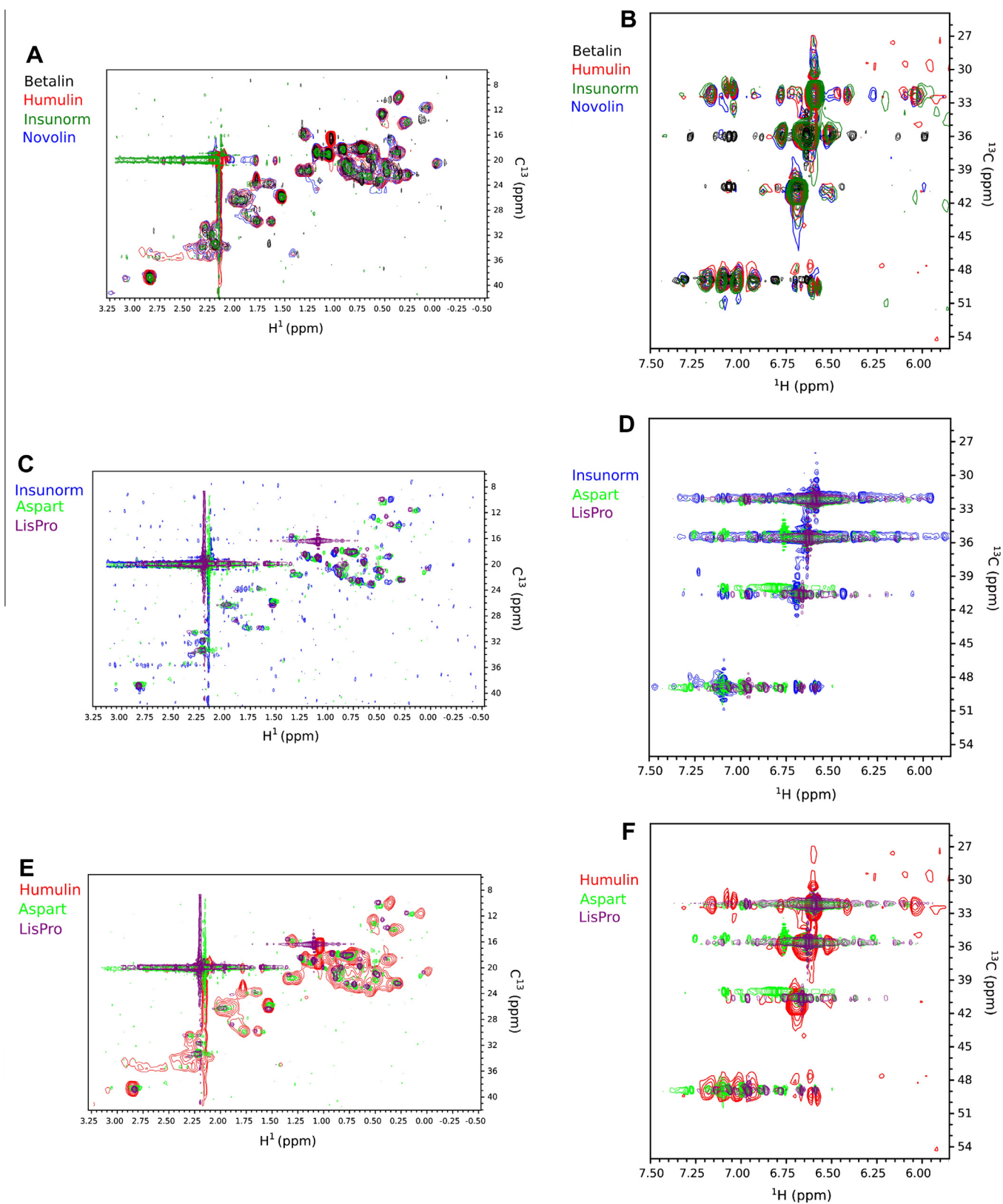


Fig. 2. ^1H - ^{13}C NOESY NMR analysis of regular insulin. We have evaluated the ^1H - ^{13}C NOESY NMR spectra of Humulin[®] R, Novolin[®] R, Insunorm[®] R and Betalin[®] H and superposed for comparison in (A) the aliphatic region and (B) the aromatic region. The specificity of the NMR fingerprint for the regular insulin was assessed by comparison of the NMR spectra of both Insunorm[®] R (C and D) and Humulin[®] R (E and F) with the insulin analogs Aspart (Asp^{B28}) and LisPro (Lis^{B28}Pro^{B29}) in the aliphatic (C and E) and the aromatic (D and F) regions. Colors are as indicated in the figure. Details in Section 2.

The SAXS data evidenced the similarity between the four insulin products (Fig. 1), suggesting equivalent molecular envelopes and oligomeric state, which is expected to be a hexamer under formulation condition [59]. The concentration dependence of the scattering profile was probed by measuring SAXS of the insulin formulation at their original concentration (100 U/mL; Fig. 1A, B, D, E, G, and H) and diluted (33 U/mL; Fig. 1C, F and I). Analysis of the SAXS data revealed the lack of effects from dilution at this concentration range on changes in the insulin scattering and thus in its conformation and oligomeric state. This behavior was equivalent for all four insulin products, for two distinct batches measured each at distinct beamlines (Fig. 1A, D and G versus Fig. 1B, E and H).

The Guinier analysis, shown as the dependence of the log of scattering intensity on the q^2 vector [40], revealed a monodisperse system as judged by the lack of any drift in the linearity, suggesting a single oligomeric form in solution and the absence of detectable aggregate (Fig. 1A–C, inset). There were only minor changes within error in main parameters such as the radii of gyration and D_{\max} (Table 4). The Guinier analysis and real space analysis obtained from the Fourier transform of the scattering profile using Gnom [41] allowed the recovery of structural parameters from the protein particle such as the maximum distance in the (D_{\max}) and the radii of gyration (R_g), which provided to be the same within error for all insulin formulations evaluated here (Table 4). The obtained pair-distribution function $P(r)$ also revealed similar molecular shape for all four formulations, both at their original formulation (Fig. 1D and E) and diluted (Fig. 1F), ruling out any concentration-dependent effects at the present analytical conditions. The Kratky plot of the scattering profile reveals a bell-shaped profile, which is characteristic of globular particles with no flexible domains (Fig. 1F–H) [40]. These behaviors were similar for the four insulin formulations at both concentrations, indicating a similarity between them in oligomeric state, particle form and globularity in solution.

3.4. Solution characterization by nuclear magnetic resonance

In order to gain insight into the overall conformation of the insulin formulations, we have performed natural abundance ^1H – ^{13}C -HSQC of these products. This approach allows the detection of all ^1H – ^{13}C chemical moieties and was previously used by other for the structural characterization of human, porcine, and bovine insulin [31,60,61]. In the spectra of the four regular insulin, the observed crosspeaks are well defined, few and dispersed, as expected for a small, well folded protein, with a sharply defined conformation rather than an ensemble of different conformational populations in solution.

The ^1H – ^{13}C -HSQC spectra of all four regular insulin are superimposable, showing similar patterns of intensity and distribution of crosspeaks in both the aliphatic (Fig. 2A) and the aromatic (Fig. 2B) regions. The signal from aromatic compounds found in insulin formulations (such as phenol and m-cresol) did not interfere in the NMR analysis, since they are found outside the spectral window reported here (<http://www.bmrb.wisc.edu/>). The methyl carbon of m-cresol has a carbon chemical shift of 23.09 ppm and a proton chemical shift of 2.28 ppm. This crosspeak is readily observable in ^{13}C -HSQC insulin spectrum, but without interference of protein signals.

In order to evaluate the specificity of the ^1H – ^{13}C -HSQC NMR measurements for probing specific conformation of the insulin products in their finished solution, we have compared the spectra obtained from one regular insulin with two insulin analogs, the Aspart (Asp^{B28}) and insulin LisPro ($\text{Lis}^{\text{B28}}\text{-Pro}^{\text{B29}}$), two engineered variants used as a fast-acting insulin. Both insulin analogs were well folded in their original solution, as indicated by their NMR spectra. Superposition of the NMR spectrum of the analogs with the regular human insulin evidenced divergences between them both in the aliphatic (Fig. 2C and E) and in the aromatic (Fig. 2D and F) regions, suggesting dissimilar conformation in solution between the regular insulin and the analogs. Collectively, these data

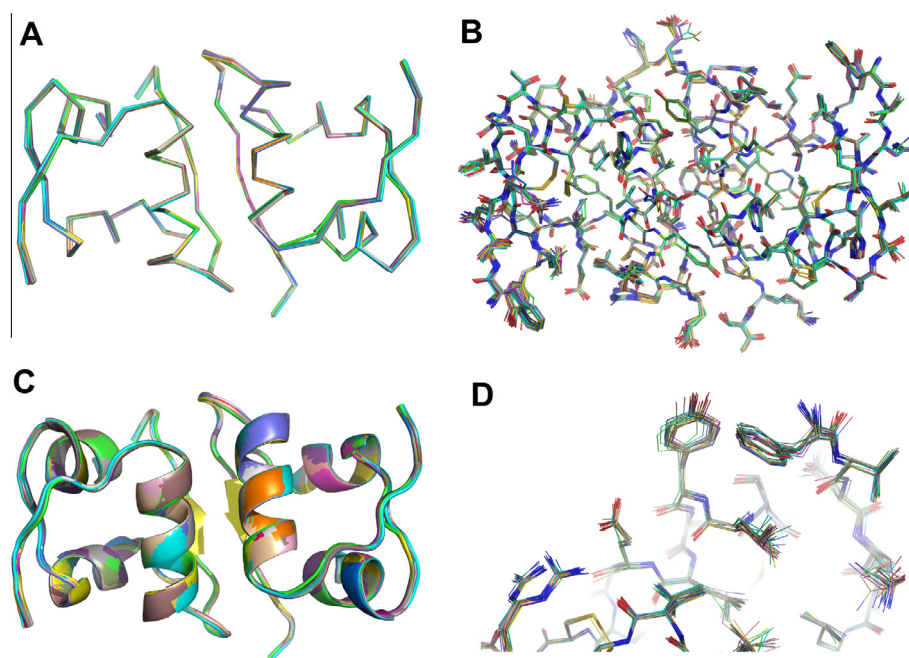


Fig. 3. Crystallographic analysis of regular insulin. The crystal structures of the human insulin from different formulations products (Humulin[®] R, Novolin[®] R, Insulin[®] R and Betalin[®] R) were solved independently from different crystal grown in varying conditions as depicted in Table 1 and superposed with PyMOL by alignment using the C α . Representation of the two insulin monomers from the asymmetric unit using (A) ribbon representation for C α backbone; (B) stick using standard atom color and (C) cartoon. Notice the conformational similarity between the 24 superposed crystal structures independently solved. (D) Detailed view of the superposition of the insulin crystal structures, showing the positional variability of the side chains at the protein surface. Details in Section 2.

Table 6

Pairwise global structural alignment of the human insulin structures. The insulin structures were superposed by pairwise global alignment using ProSMART [51], and the RMSD was calculated and reported in Table 5. The mean value and standard deviation for the RMSD for each subgroup as defined in Table 5 is reported here.

	Betalin	Insunorm	Novolin	Humulin
Betalin	0.21 ± 0.06	0.21 ± 0.08	0.25 ± 0.08	0.26 ± 0.09
Insunorm		0.16 ± 0.06	0.30 ± 0.11	0.28 ± 0.11
Novolin			0.29 ± 0.10	0.25 ± 0.13
Humulin				0.22 ± 0.17

tive (5–10%). Minor positional variations of human insulin side chains were observed, mainly in the protein surface (Fig. 3B and D), revealing a larger dynamics of the protein at this region. All crystal structures of the regular human insulin were found with the two insulin monomers of the asymmetric unit in the “T” state, i.e., with the region B1 to B8 in an extended conformation (Fig. 3C).

The insulin structures show also two zinc ions, each coordinated by the His^{B10} from each insulin monomer and by additional chloride ions (not shown). In one (4FG3.pdb) of the crystal structures reported here, we were able to model a glycerol molecule, which was found lying between the His^{B5} and the Gln^{B4} (not shown). We were not able to find any electron density blob compatible with either metacresol or phenol in any particular region of the crystallographic protein structure. No other ligand could be modeled in the remaining electron density map.

Further attempts to grow crystals from the insulin analogs Lis^{B28}-Pro^{B29} (insulin LisPro) and from Asp^{B28} (insulin Aspart) pharmaceutical products using the above mentioned crystallization conditions were unsuccessful (not shown). The failure in crystallizing the insulin analogs using the same experimental approach applied for the regular human insulin indicates that only minor association and packing effects during the crystallization process would be governing its final conformation. Altogether, these data indicate that the present crystallization method reported here are sufficiently robust and specific as a bioanalytical method for the determination of the high resolution structure of wild-type human insulin direct from pharmaceutical formulations.

4. Discussion

The first insulin crystal structure has been obtained about 50 years ago [67], since the first attempts decades before [68], by using the traditional batch annealing crystallization technique [69], comprising heating and cooling of insulin solution. Since then, spectroscopic and crystallographic techniques have been applied to exploit the structure and conformational transition in insulin and their correlation with biological activity [33,57,70–77]. More recently, X-ray powder-diffraction from microcrystalline material has also been shown to be effective in the elucidation of insulin structure [28] and also accurately used in the determination of dissimilar polymorphic forms of insulin formulations [78,79].

Since the seminal description of the production of insulin and its therapeutic use in the management of diabetes [14], several insulin products have been developed, and heterologous insulin has been mostly replaced by human insulin produced from distinct methods and formulated with varying excipient composition, either by recombinant expression in *E. coli*, *S. cerevisiae*, and *P. pastoris* or by semi-synthetic preparation from porcine insulin [80–86]. The large number of variables in the production of therapeutic biological agents can impact directly over their quality, efficacy, and safety [20,87,88]. Relying on the protein structure–function paradigm, these parameters are intrinsically related to the chemical and structural integrity. In this context, while many pharmaco-

peical analytical and bioanalytical methods are available for the quality assessment of insulin products, it is highly desirable exploiting the three-dimensional structure of protein in its final formulations in order to ensure the appropriate folding and quaternary assembly and thus the biological function. The use of conformational and high-resolution structural information has been suggested as a requirement for the comparability evaluation of biological follow-on products [89–93]. However, to date no high-resolution structural requirement is made for human insulin in the current US, European, Japanese, and Brazilian pharmacopeias. Instead, the biological and, indirectly, the structural integrity of insulin are indirectly accessed through biological assays, with the single-point evaluation of the glycemia lowering efficiency by insulin products, along with other biochemical proofs of purity and potency.

In the present study, we have made the combined use of modern state-of-the-art structural techniques (mass spectrometry, dynamic light scattering, small-angle X-ray scattering, nuclear magnetic resonance, and protein crystallography) for the detailed characterization of the chemical and structural integrity, accessing the correct folding through the evaluation of the secondary, tertiary, and quaternary structural arrangement of the human insulin in four distinct pharmaceutically available products from varying brands, differing completely in formulation composition, expression system and purification procedures, formulated to the same potency.

We have achieved a satisfactory crystallization approach which combine technical feasibility (as shown by the crystallization method: vapor diffusion and simple precipitant composition), reproducibility and robustness (as shown by solving their structures from crystals obtained from varying batches of both insulin and chemicals, crystal grow temperature, different producers, X-ray sources, detectors, freezing methods, and different analysts), and by its selectivity (as shown by the lack of crystallization of two insulin analogs, aspart – Asp^{B28} – and LisPro – Lis^{B28}Pro^{B29}). The present crystallization approach resulted in good diffracting quality crystals within a few days for all regular human insulin products, both presenting same space group, similar cell parameters and conformation. All structures consist of two insulin monomers and two zinc atoms per asymmetric unit coordinate by the His^{B10}. The overall conformations of both insulin monomers are similar, with C α RMSD of less than 0.3 Å.

All the 24 crystal structures reported here were in the T(6) state, i.e., with the six monomers of the hexamer in the T state, with the B1 to B8 segment in extended form. This conformation is typical of the insulin in the absence of phenolic compounds, while the R state is achieved by the interaction with phenolic compounds. The concentration of phenolic compounds and zinc in the formulations studied here are supposed to be sufficient to achieve saturation in the T → R transition [94] at pH 7.4. However, the present crystallization procedure used here involves the use of buffers at pH 5.5 and other components of the mother liquor. These variables could diminish the free energy of transition from the R to the T state, resulting in the T(6) conformer observed in the crystal structures reported here. At present, it is not possible to precise the mechanisms for this finding. Although the crystallization process might introduce some sort of drift in the conformational transition of insulin, the crystallization procedure seems to be well selective for the present conformer observed here for the regular insulin, since the insulin analogs Aspart and LisPro could not be crystallized using the same approach.

Along with the crystallographic evidences for the structure of insulin obtained from the final formulated products, NMR could also be a useful reference for crosspeaks arising from the aromatic carbons. Differences in primary structure can be precisely detected in these NMR experiments. Also, a fingerprint of the primary struc-

ture and global folding can be generated [95,96], enabling a rapid assay to compare different products or to monitor the chemical and folding integrity of insulin samples. The 2D-NMR spectra can be used as “fingerprints” for topological characterization of insulin in the final pharmaceutical formulation, sensible enough to detect subtle differences as mutations, different side chain packing, or small differences in folding. These data support the use of ^1H – ^{13}C -HSQC NMR spectra as a survey for the insulin conformation in solution, as a conformational fingerprint. NMR and crystallography are very complementary techniques in the elucidation of the high resolution protein structure [31,56,97–100]. The combination of crystal structure from single crystals obtained from final formulation and the NMR fingerprints can assist in further routine analysis of insulin products and formulation studies.

Government initiatives at several nations have motivated the development of biological follow-on products including insulin [90–93,101–103] which has allowed the approval of several products in the European Union Health Agency since 2005 and one in Brazil [104–107]. Complimentary to the use of the present structural approaches for quality assessment of insulin in its final formulated product, these data could be used in the structural comparability exercises of insulin products, which in special given the fact that they all share the very same chemical identity. Moreover, it has been reported the similarity in the biological activity of regular acting human insulin products *in vivo* [108,109]. Other studies have suggested the need of extensive characterization for biosimilar [110,111]. However, we understand that similar conformation does not preclude exact biological activity, since distinct formulation could modify protein properties in the administration site and beyond, such as the kinetic release, absorption, and interaction with molecular partners [74,88,108,112–116]. Additional biological and non-inferiority clinical evaluation should be addressed in conjunction with structural information in comparability studies.

These combined methods can assist in the determination of chemical integrity (mass spectrometry), solution conformation (NMR fingerprint), and structural integrity (NMR and X-ray crystallography) of regular acting human insulin in final formulations. These structural methods are currently well-established, and they can be accessed in most countries, in special those for the main pharmaceutical markets, the Americas, Europe and Japan, whose pharmacopeias were above mentioned. It could be used in routine evaluation of structural integrity and identity, as a part of current or evolving methods aiming the minimization of animals' requirement in routine quality control, in the development of novel insulin products, or in future protocols for a thorough comparability exercises between follow-on protein product and a reference product. We believe that the information gathered in the present study would assist in further establishment of biological structural standards for routine pharmacopeical and metrological quality analysis.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejpb.2013.05.005>.

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