NMR Spectroscopic Analysis to Evaluate the Quality of Insulin: Concentration, Variability, and Excipient Content

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Abstract

Known and consistent bioactivity between samples of insulin is essential to correctly estimate the dose. Insulin concentration is not the same thing as bioactivity, however, and methods to correctly determine both are required. Here we show that one dimensional nuclear magnetic resonance (1D NMR), in contrast to, for example, reverse phase high pressure liquid chromatography, can be used to determine both insulin concentration as well as confirm the structural integrity required for activity. In response to the report by Carter and Heinemann, we decided to investigate insulin intended for public use. Insulin from several manufacturers was investigated. Correct insulin concentrations were found in all tested samples although the general sample variability, which possibly could influence the bioactivity, varied depending on insulin type and manufacturer.

Diabetes mellitus type 1 (T1D) is a global disease with growing incidence.[1] Diagnostic criteria are based on laboratory measurement of plasma glucose levels and the presence of symptoms.[2] Inadequate insulin treatment to replace the declining endogenous insulin secretion from the beta cells in the pancreas leads to abnormalities of carbohydrate, fat and protein metabolism, and risk for development of acute and long-term complications and eventually death. The quality assurance of the insulin delivered is a cornerstone for successful treatment. Recently Carter and Heinemann[3] questioned the accuracy of the content of insulin, which in vials and cartridges should be 95-100 U/ml upon release (USP/FDA). Randomly acquired multidose human insulin vials were analyzed by an LC-MS approach. Eighteen 10 ml regular and NPH-insulin vials, representing 16 different insulin lots from two manufacturers collected from five pharmacies, were analyzed. Surprisingly, the intact insulin concentration ranged from 13.9 to 94.2 U/ml, with a mean of 40.2 U/ml. No vial met the minimum standard of 95 U/ml. The conclusion was that these results implied that the cold supply chain impacts insulin concentrations to a larger extent than anticipated. The Carter and Heinemann research evoked a lot of questions, both on the accuracy of the measurements presented but also on the reliability of the delivery of insulin from the manufacturers to the pharmacies. The next step, from the pharmacy to the hospital ward or directly to the patient, was not investigated. Subsequently, research from Novo Nordisk describing the QA of a much larger sample set of their insulin batches spanning ten years was published in

response to Carter and Heinemann's findings.[4] Using a reverse-phase HPLC approach, they show that in no case were there any insulin sample with less than the minimum standard of 95U/ml.

Nuclear magnetic resonance (NMR) spectroscopy is the golden standard in structure elucidation of small organic molecules. It is also used for mixture analysis and metabolomics due to the inherent quantitative aspects of the technique. The information extracted from NMR is more extensive than, for example, reverse phase HPLC methods. Even in the 1D NMR case which is swiftly recorded in the time-frame of a few minutes per sample it provides not only absolute quantification of multiple mixture components by simply integrating their corresponding peaks which are proportional to their concentrations. It is also very sensitive to changes of, for example, peptide secondary and tertiary structure, peptide interactions, and pH or ionic strength variations, all seen as perturbations in the chemical shifts, that is, slight but notable changes of peak positions along the x-axis. Previously,

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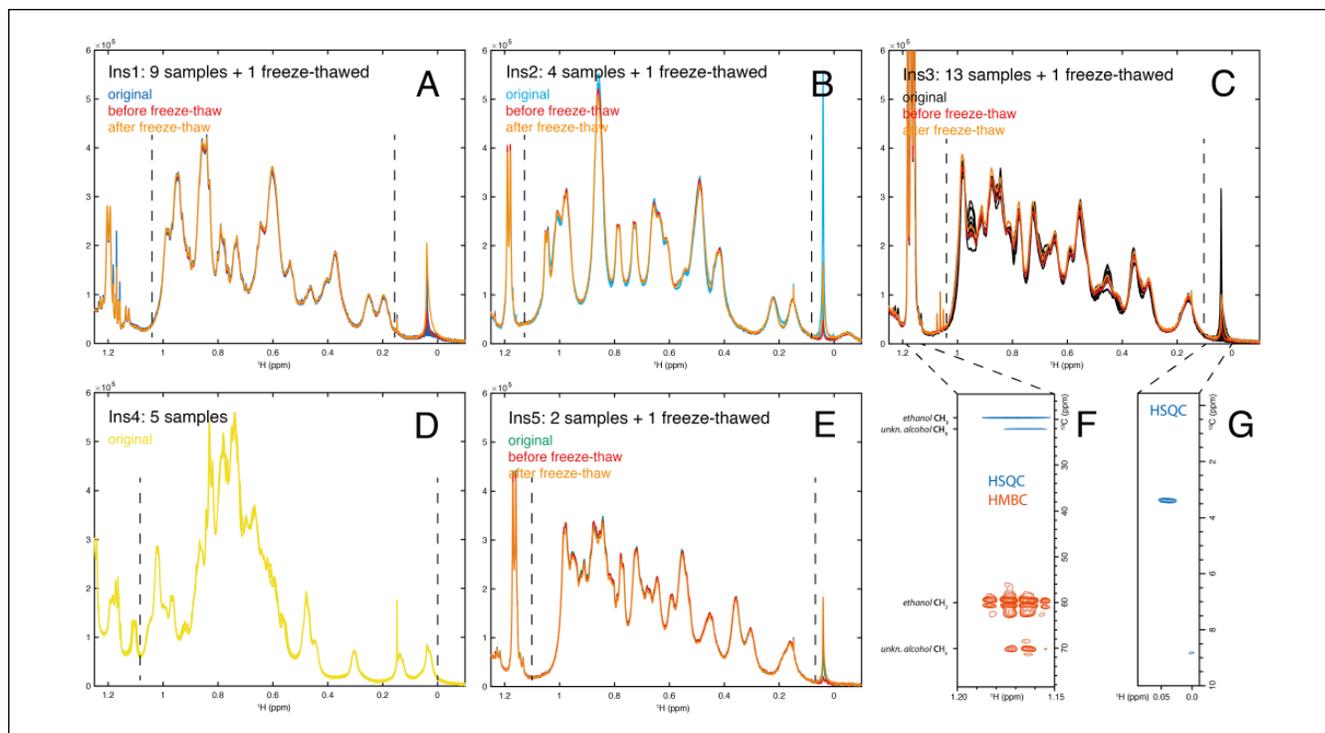Email: daniel.malmodin@nmr.gu.se

Figure 1. (A-E) The limits between which the integral was used to calculate the concentration of insulin is marked in each formulation by dotted lines (Table 1). The Ins3 spectra show fairly large variation compared to Ins1, Ins2, and Ins4. (F and G) Using 2D ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC spectra ethanol could be identified while the origin of the peak at 0.04 ppm (^{1}H) and 3.6 ppm (^{13}C) remains unknown.

natural abundance heteronuclear NMR has been used to characterize the structural integrity, potential degradation, and variability on an amino acid level for insulin.[5,6] The aim of the present study was to evaluate 1D ^{1}H NMR as a method for rapid and robust characterization of insulin in terms of insulin concentration, variability, and excipient content in as close to a native state as possible. Also, in light of the Carter and Heinemann findings,[3] a comprehensive study of five of the most common commercial insulins, not directly from the manufacturer, but rather about to be delivered or already in the hands of patients, was performed.

Materials and Methods

In total 29 vials/cartridges of three different short acting insulin analogs, one rapid acting insulin, and one basal insulin analog were collected from 7 different wards at the Queen Silvia Children's hospital, Gothenburg, Sweden. In addition, four vials of short acting insulin analogs were collected from patients, that is, from the home of children with diabetes. All vials and cartridges were carefully transported and stored under continuously monitored refrigerated conditions between 2 and 8°C after collection until analyzed. The lots were cataloged by date and place of acquisition, batch number, and expiration date. Expiration dates ranged from October 2018 to March 2020 and repeated analyses were performed during the time period April 1, 2018 to July 11, 2018.

NMR Spectroscopy and Analysis

Insulin NMR samples were prepared by mixing 540 µl of the respective insulin formulation with 60 µl D$_2$O (CortecNet, Voisins-Le-Bretonneux, France) before transfer to 5 mm SampleJet NMR tubes (Bruker BioSpin, Rheinstetten, Germany). 1D ^{1}H NMR data were acquired on a Bruker Avance III 600 MHz spectrometer equipped with a room temperature 5 mm BBI probe and a cooled SampleJet automatic sample changer with the standard pulse sequence "noesygp-pr1d," including addition of an ERETIC signal for quantification purposes.[7] The integrals of mannitol signals in a quantification reference sample supplied with the Bruker IVDr platform (https://www.bruker.com/products/mr/nmr/avance-ivdr/overview.html) were used as ERETIC reference. Samples were kept in the SampleJet at 6°C before data acquisition. Some samples were kept in room temperature for several weeks and one Ins3 sample was kept at 37°C for nine days before repeating data acquisition. Data were processed and integrated using TopSpin3.5pl7 (Bruker BioSpin, Rheinstetten, Germany) and referenced to the glycerol C-H chemical shifts of the human metabolome database entry HMDB00131.[8] STOCSY[9] was performed with an in-house script implemented in MatLab (Mathworks Inc, Natick, USA). To quantify insulin, the methyl region of the spectra (Figure 1) was integrated and assumed to constitute only insulin-derived signals. With the chemical shift assignments
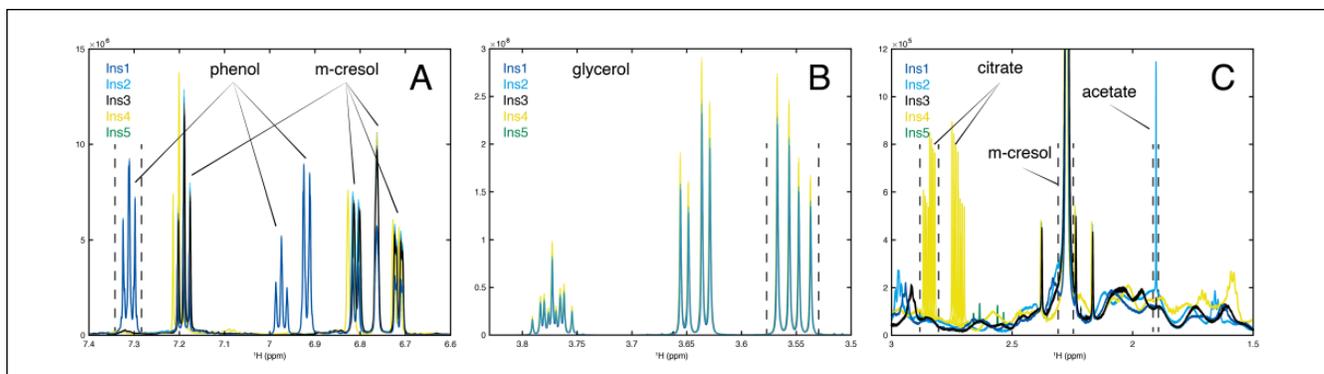
Figure 2. Three selected regions of the NMR spectra of all 33 insulin samples displaying major excipient signals. The limits between which the integrals were used to calculate the respective excipient concentration (Table 1) are marked with dotted lines.

for human insulin as basis,[10] the region chosen for integration encompasses signals from 75 protons (24 methyls, two Ile H γ and one Leu H β). Given that all chosen insulin formulations contain the same number of protons, the concentration was calculated from relating the integral to the ERETIC signal, 75 protons, and the dilution factor resulting from D_2O addition. 2D $^1H,^{13}C$-HSQC and $^1H,^{13}C$-HMBC spectra were acquired for a sample of the Ins3 insulin formulation using the standard pulse sequences "hsqcedetgpsisp2.3" and "hmbcetgpl3ndpr," respectively. Excipient concentrations were calculated using a similar ERETIC approach as for insulin (Figure 2). Specific parameters for the NMR data acquisition and processing is available upon request.

Sample Mistreatment

Additional aliquots of four insulin samples were deliberately mistreated by repeated freeze-thaw cycles of −20°C for 45 min followed by thawing at 37°C on an Eppendorf Thermomixer with shaking at 800 rpm for 15 min, for a total of three cycles.

Results and Discussion

As has already been reported, 2D NMR is an excellent tool for characterization of insulin on an atomic level.[5,6] However, the use of (quantitative) two dimensional natural abundance $^1H,^{13}C$-HSQC NMR spectroscopy typically requires several hours even when using a modern cryoprobe on a high field (>700 MHz) NMR spectrometer. Here, we report the use of standardized 1D ^1H NMR on a standard 600 MHz instrument for rapid assessment of as close as possible native state insulin, including quantification and excipient characterization. The ability to acquire 1D data in a few minutes is a distinct advantage for characterization and quality control purposes. The chemical shift of every amino acid proton is sensitive to the chemical environment and reports on both local and global changes. This also applies to methyl groups, often buried in the interior of a folded soluble protein such as, for

example, insulin. In the present study, the structural integrity is judged from the signal of all isoleucine, valine, and leucine methyl groups, representing nearly a quarter of all amino acids in insulin, and indirectly also comprising the effect from several aromatic side chains being part of a common hydrophobic core of the protein. When the structural integrity is not compromised, the concentration is accurately determined from the sum of the signal intensities in the rightmost part of the methyl region (ie, between 1.0 ppm and 0 ppm).

We could not reproduce the high range in concentration variability reported by Carter and Heinemann. In fact, the sample concentrations from all manufacturers were remarkably stable with no signs of variation even after keeping samples several weeks at room temperature, or, for one Ins3 sample, nine days at 37°C (data not shown). In accordance with what was reported in Moses et al,[4] all our samples had insulin concentrations within specification, that is, between 98 to 107% of 3.5 mg/l, as stated in the manufacturer leaflets of the different types (Table 1). Since there was no observable change in insulin concentrations, we chose to deliberately mistreat four samples by repeated freeze-thaw cycles to provoke insulin degradation or general detrimental effect on the formulation. But even for these samples, the insulin concentration was within specification for all four samples. However, although the concentration, determined from integrating the methyl signals, is almost invariant between batches for all insulin types, it is clear from the 1D NMR spectra that there is some variation in the formulation, at least in the Ins3 samples (Figure 1C). The sample set only included two Ins5 samples and thus no variability estimate of these can be made, but Ins1, Ins2 and Ins4 shows very high similarity within each group. It might be that the Ins3 variability originates from the factory or that Ins3 formulation is more sensitive compared to Ins1, Ins2, or Ins4 since no sample obtained from patients stood out compared to the ones from the hospital and it is unlikely that only Ins3 could have been maltreated at the hospital without the same thing occurring for Ins1, Ins2, or Ins4 obtained from the same places. We have not tried to elucidate what these observed small

Table 1. Sample Overview With Insulin and Excipient Concentrations.

Sample/type	Expiry date (no of samples)	n	Freeze-thaw	[Insulin] mM ± SD (mg/ml ± SD)	% of 3.5 mg/ml Insulin	[Glycerol] mM ± SD	[m-Cresol] mM ± SD	Other excipients concentration ± SD
Ins1/Rapid-acting analog	02/2019 (3), 07/2019 (3); 10/2019 (1); 01/2020 (2)	9	No	0.593 ± 0.0022 (3.44 ± 0.013)	98	215 ± 0.53	17.5 ± 0.13	Phenol 15.6 ± 0.065 mM
	01/2020	1	Yes	0.596 (3.45)	99	215	17.6	Phenol 15.6 mM
Ins2/Short-acting	10/2018 (2); 12/2018 (2)	4	No	0.604 ± 0.019 (3.50 ± 0.11)	100	223 ± 3.2	31.1 ± 0.46	Acetate 154 ± 7.0 μM
	10/2018	1	Yes	0.577 (3.34)	96	224	31.1	Acetate 161 μM
Ins3/Rapid-acting analog	10/2019[a] (4); 10/2019[a] (5); 11/2019 (1); 01/2020 (1); 02/2020 (1); 03/2020 (1)	13	No	0.623 ± 0.014 (3.61 ± 0.083)	103	217 ± 1.1	31.2 ± 0.091	Ethanol[b] 5.29 ± 0.14 mM
	10/2019	1	Yes	0.648 (3.76)	107	217	31.2	Ethanol[b] 5.41 mM
Ins4/Basal analog	04/2018 (1); 04/2019 (1); 05/2019 (1); 10/2019 (1); 03/2020 (1)	5	No	0.646 ± 0.0097 (3.74 ± 0.056)	107	253 ± 3.3	28.3 ± 0.31	Citrate 459 ± 46 μM
Ins5/Rapid-acting analog	01/2020 (2)	2	No	0.608 ± 0.010 (3.53 ± 0.059)	101	218 ± 2.1	31.8 ± 0.42	Citrate 48.2 ± 7.4 μM
	01/2020	1	Yes	0.597 (3.46)	99	218	32.2	Citrate 56.4 μM

[a]Two different batches. [b]Major fraction of methyl triplet (see Figure 1F).

differences are in terms of chemical shift perturbations of the individual amino acid methyl groups. Whether the variability in the formulation affects the bioactivity is impossible for us to say but is not inconceivable.

After the repeated freeze-thaw procedure, precipitation was observed by visual inspection in two (Ins3 and Ins5) of the four selected samples. Surprisingly, after resuspension, transfer to NMR tubes and incubation at 6°C overnight, the precipitate disappeared, suggesting spontaneous redissolution. Whether precipitation was responsible for the perturbation seen in the Ins3 spectrum is difficult to say and the freeze-thaw experiment was not repeated to check the contents of only the supernatant. It is not possible to single out any specific insulin formulation as being less stable than the others from the minimal tests reported here, but the observed conformational change of Ins3 after three freeze thaw cycles shows the importance of not storing insulin too cold also during short periods. It is clear that in general it is better to store insulin at room temperature for periods of days if refrigerator alternatives at hand are too cold.

The Ins3 samples include an unlisted excipient, ethanol, with a concentration in the same range (mM) as the other additives. Two other unlisted excipients were readily quantifiable in Ins2 (acetate) and Ins4 and Ins5 (citrate) but at much lower concentrations. The addition of a cation to Ins4 formulation is evident as the citrate doublets shift between the different batches, also highlighting that there is a certain degree of variation between batches. A singlet signal at 0.1 ppm has previously been associated with monomeric

insulin,[11] possibly the same signal as seen in the present data at chemical shifts of 0.039 ppm and 3.36 ppm for ^{1}H and ^{13}C, respectively (Figure 1G). In samples subjected to freeze-thaw cycles this signal increased after the maltreatment (Figure 1). In contrast, the signal decreased markedly in the Ins3 sample kept at 37°C for nine days (data not shown). Whether the variation in this signal actually reflects the abundance of monomeric insulin is very unclear as there is no correlated or anticorrelated change in the rest of the spectrum as judged by STOCSY,[9] or other peak shift, intensity, or shape changes supporting this by visual inspection. Also, no additional follow-up studies on this particular peak were made.

We here show how 1D ^{1}H NMR spectroscopy, combined with minimal preanalytical sample preparation on insulin samples intended for immediate use, can be used to rapidly detect and characterize concentration changes, assess protein structure perturbations, as well as determine the additive content. Our finding provokes the question why the established quality control method is reverse-phase HPLC where only the concentration can be assessed. Given that the certified RP-HPLC method employed in Moses et al[4] involves acidification and denaturing conditions, any structural information apart from correct disulfide linkage is lost and possibly any precipitates also redissolved. Therefore, the concentration reported is a total concentration, not necessarily the concentration of bioactive insulin. RP-HPLC would never be able to ascertain the perturbations seen in the 1D NMR approach presented here.

Given the findings in Moses et al[4] and the results presented here, despite some batch variation in one insulin from one manufacturer, there does not appear to be any acute problems in the distribution or longevity of insulin, pointing toward the results in Carter and Heinemann[3] being a consequence of issues with the employed analytical method.

Abbreviations

1D, one dimensional; 2D, two dimensional; ^{1}H, proton; FDA, US Food and Drug Administration; HPLC, high pressure liquid chromatography; LC-MS, liquid chromatography–mass spectrometry; NMR, nuclear magnetic resonance; NPH, neutral protamin hagedorn; QA, quality assurance; T1D, type 1 diabetes mellitus; USP, US Pharmacopeia.

Declaration of Conflicting Interests

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