Published in final edited form as:

J Pharm Sci. 2015 April ; 104(4): 1555–1560. doi:10.1002/jps.24330.

Analyzing Insulin Samples by Size-Exclusion Chromatography: A Column Degradation Study

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Abstract

Investigating insulin analogs and probing their intrinsic stability at physiological temperature, we observed significant degradation in the size-exclusion chromatography (SEC) signal over a moderate number of insulin sample injections, which generated concerns about the quality of the separations. Therefore, our research goal was to identify the cause(s) for the observed signal degradation and attempt to mitigate the degradation in order to extend SEC column life-span. In these studies, we used multi-angle light scattering (MALS), nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC/MS) methods to evaluate column degradation. The results from these studies illustrate: i) that zinc ions introduced by the insulin product produced the observed column performance issues; and ii) that including EDTA, a zinc chelator, in the mobile phase helped to maintain column performance.

Introduction

A recent study using size-exclusion chromatography (SEC) to assess the stability of three different insulin analogs containing various phenolic preservatives was reported [1]. In these studies, Teska *et al.* observed a rapid decrease in the SEC column performance; in fact, it only required a moderate number of sample injections (approximately 80–100) to produce this observation [1]. These observations were corroborated via conversations with other researchers who extensively perform insulin research and analysis [2, 3]. Given the large amount of insulin and insulin analogs produced worldwide, and the considerable cost of the SEC columns, we felt that these column performance issues warranted further investigation and are the scope of the current work.

Since many diabetics require multiple injections per day in order to maintain an acceptable blood glucose level, marketed insulin and insulin analogs are formulated in multi-dose vials [4]. Furthermore, multiple needle insertions into a sterile drug product vial inherently increase the possibility of bacterial contamination. The FDA (Food and Drug Administration) requires drug products in multi-dose vials to include an antimicrobial preservative. In the case of insulin formulations, phenol and/or meta-cresol are commonly employed. Insulin is somewhat of a special case, as it is well established that the presence of phenol and/or meta-cresol promotes favorable conformational changes in the insulin

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hexamer form [5, 6] and provides added stability to the drug product [7, 8]. It is also well known that insulin, and insulin analogs, exhibit a complex self-assembly process to produce hexamers coordinated by two zinc ions [9]. This assembly confers additional stability [1, 10, 11, 12, 13] providing a more robust shelf-life, but it is important to note that the monomer is the pharmacologically active unit [14]. When we inject an insulin sample onto a SEC column, we assume – due to the concentration dependence of insulin self-association [15] – that the dilution into the column's flowing mobile phase intrinsically triggers the insulin hexamers to dissociate and ultimately release bound zinc ions (Zn^{+2}) and the various phenolic additives. Based on observations by Teska *et al.* [1], we hypothesized two fundamental explanations for the observed SEC column performance issues: i) either the phenolic preservatives were accumulating on the silica, creating a more hydrophobic surface; and/or, ii) the released zinc ions were modifying (reacting with) the silica end caps resulting in a modified surface (i.e. –Si-O-Capped surface to produce –Si-OH groups). Either condition would result in degraded column performance, as they both would introduce unwanted modes of interaction between analytes and the column resin.

Materials and Methods

Materials

Insulin lispro (Humalog Lot: CO74516A; Eli Lilly, Indianapolis, Indiana) was purchased from a local pharmacy. All laboratory chemicals used were analytical grade or higher. Insulin lispro and all chemicals were used before their expiry date. Water used in mobile phases, formulations and buffers was purified through a Millipore Synergy UV (Millipore, Billerica, MA) filtration unit (MilliQ). Deionized (DI) water was used to rinse the liquid-liquid extraction sample vials.

Silica Resin Incubation

Tosoh G2000SWXL top-off resin (2.0 g; Tosoh, King of Prussia, PA) was gently shaken to suspend the resin in the manufacturer storage solution and pipetted into a 9 mL glass test tube. The tube was centrifuged (2000 g, 5.0 min) and the supernatant was discarded; the resin was rinsed by adding MilliQ water (3.0 mL) to the settled resin and re-suspended by gentle shaking. For each sample, this process was repeated three times to ensure full removal of the storage solution. After the final rinse, the supernatant was discarded and 3.0 mL of either MilliQ water; 147 μM ZnCl_2 in water; 147 μM ZnCl_2 and 440 μM EDTA in water; or a 14 mM Na_3PO_4 , 174 mM glycerol, 30 mM meta-cresol in water (referred to as lispro buffer) was added. The tubes were capped and shaken to re-suspend the silica resin. Samples were incubated (18–22 °C; 24 hr) on a test tube rotator set to 40 RPM to ensure adequate suspension of the resin during the incubation period. Used resin was removed from degraded columns from the previous stability study [1]; columns were unpacked using a spatula and the dry unpacked resin was slurried in the smallest possible volume of MilliQ water to produce a mixture which could be easily pipetted (approximately 3.0–5.0 mL). No further treatment was implemented on these samples.

Liquid-Liquid Extraction

Used and treated resin samples were extracted by adding suspended resin slurry (approximately 5.0 mL) to a separatory funnel (250 mL), ethyl acetate (EtOAc; 2 vol) was added to the funnel, capped and vigorously shaken. After settling, the aqueous-slurry layer was removed and saved. The organic layer was transferred to a flask and the extraction process was repeated three times. The organic layers were combined, dried over anhydrous MgSO_4 , filtered and concentrated under reduced pressure (*i.e.* rotary evaporation). The residue weight was determined and the sample reconstituted in dichloromethane (1.0 mL) and analyzed by GC/MS, or diluted in CDCl_3 (1.0 mL) and analyzed by NMR.

Size-Exclusion Chromatography

SEC was performed on an Agilent 1100/1200 HPLC (Agilent Technologies, Santa Clara, CA) detecting UV absorbance (280 nm). Multi-angle light scattering (MALS) measurements were made with a Wyatt DAWN EOS 18-angle MALS detector (Wyatt Technology Corp., Santa Barbra, CA) plumbed in series with the UV detector. Molecular weight calculations were done in Wyatt's ASTRA software (version 5.3.4). The mobile phase was degassed and filtered (0.2 μm filter) before use. Samples were centrifuged at (9650 g, 10 min) before injection (50 μL) to remove large insoluble aggregates. A Tosoh TSK-gel G2000SWXL SEC column (7.8 mm $\times$ 300 mm; Tosoh Corp., King of Prussia, PA) was employed at a flow rate of 0.5 mL/min using 0.3 M NaCl, 0.1 M Na_3PO_4 , pH 7.4 with and without ethylene-diamine-tetra-acetic acid (EDTA; 440 μM) as the mobile phase (MP); the HPLC run time was 54 min. A saturated uracil-water solution (24 μL) was added to bovine serum albumin (BSA) (1.0 mL, 2.0 mg/mL; Thermo Scientific, Rockford, IL) and used as our SEC reference standard, and referred to as BSAU. The injection sequence used was as follows: BSAU, 1 injection; insulin lispro, 8 injections; BSAU, 1 injection. This sequence was repeated (24 times) for each mobile phase (MP) condition (with and without 440 μM EDTA) resulting in a total of 192 insulin lispro injections and 48 BSAU injections per mobile phase condition. Samples were stored in amber HPLC vials in the auto-sampler at room temperature (18–22°C) over the nine day experimental analysis duration. These conditions were well within the acceptable temperature conditions and time limits on the prescribing information for insulin lispro [16]. Data were exported from Agilent's ChemStation software and analyzed with custom scripts written in Matlab and Perl.

Nuclear Magnetic Resonance and Gas Chromatography-Mass Spectrometry

The ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded using a 400 MHz Bruker NMR, Avance III 400 in CDCl_3 containing tetra-methyl-silane (TMS; $\delta = 0.0$) as an internal standard. Gas chromatography-mass spectrometry (GC-MS) was performed on a Shimadzu GC/MS-QP2010 Plus gas chromatograph mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). The GC2010 was equipped with an Rtx-5MS column (0.25 μm thickness, 30.0 meter, and diameter of 0.25 mm). The settings were as follows: column temperature was initially 40 °C with an injector temperature of 275 °C; the temperature was held at 40 °C for 4.0 min and then ramped at 10 °C/min to 280 °C, and then to 300 °C at 2°C/min and held for 18 min. The ion source temperature was set at 250 °C and the interface at 275 °C. Mass was scanned from 50 – 750 m/z from 7.0 – 56.0 min with a

scan speed set at 5000. High grade helium was used as the carrier gas and operated in the split-less mode with a pressure of 70.1 kPa, total flow of 14.4 mL/min, column flow was 1.03 mL/min, linear velocity was 36.7 cm/sec, and a purge flow of 3.0 mL/min. Final data were baseline corrected using an asymmetric least squares method implemented in Matlab [17].

Results and Discussion

Size-exclusion chromatography is an important analytical technique but one requires robust methods in order to meet the demands of research and quality/process control. In brief, SEC separations occur fundamentally based upon the analyte's accessible volume. Columns are packed with silica particles that have a characteristic size and porosity. Large analytes, for example, are too big (bulky) to enter into the silica particle pores and, as a result, are not readily retained by the column and elute first. Smaller analytes may enter or interact with the pores, which result in longer elution times. Manipulating the overall particle and pore sizes, a researcher may alter the column features to obtain a desired separation. Since silica particles have hydroxy (Si-OH) groups, many SEC column manufacturers use proprietary techniques to chemically derivatize and "cap" the hydroxy ends of the silica particles (i.e. – Si-O-Capped); a process performed with the intent to avoid ionic or hydrogen bonding interactions with the analytes [18, 19, 20]. Generally, this capping eliminates many unfavorable column-analyte interactions, but, in practice, it is very difficult to fully eliminate the effect of exposed surface charge. As a result, researchers will commonly add salt(s) to the mobile phase in order to assist in shielding the analyte from the charged uncapped groups on the surface of the silica [21, 22, 23, 24, 25]. This requirement can be problematic, as additional salt can alter aggregation or assembly state of the analyte and confound the SEC analysis [26]. Therefore, when one develops an SEC method, one can spend a significant amount of time optimizing the mobile phase conditions.

In the current study, we further investigated insulin formulations via SEC analysis. In a previous study, insulin lispro reference samples were injected onto the SEC column and used to monitor column performance throughout the study; these data are shown in Figure 1. These reference samples were stored in the product vial at $4 \pm 1^\circ\text{C}$ and injected once for every eight experimental insulin sample injections. After approximately 80 total injections, column performance issues were apparent (Figure 1; yellow traces) and clearly visible after approximately 100 insulin injections (Figure 1, red traces) [1]. As the injection number increased, the main insulin peak migrated to a longer elution time and developed a post-main peak shoulder ultimately becoming a separate peak (24–27 min). Reversed-phase chromatography (RPC), run in conjunction with SEC during this study, showed no fragments in these samples (data not shown), and was therefore consistent with the notion that the TSK-G2000SWXL column was being degraded during the study. As discussed in the introduction, since commercially available insulin formulations contain phenolic preservatives and zinc ions, we speculated that one or both of these may be triggering the observed SEC column performance issues.

To confirm that dilution upon injection of insulin lispro onto the SEC column resulted in the dissociation of hexamers into a smaller-order species – thereby releasing bound zinc ions

into solution – we measured multi-angle light scattering (MALS) during column elution (Supplemental Figure 2). The main insulin peak eluted with a weight-average molecular weight of 14.2 ± 0.3 kDa; results which support the notion that during elution on the SEC column, insulin lispro was minimally assembled and no longer tightly bound the zinc ions found in the hexamer; freeing them to enter into the solution.

We unpacked and extracted resin (see materials and methods section) from used columns, which displayed the observed SEC column performance issues. The extract from unused (*i.e.* new) versus used resin were analyzed via classical NMR methods. Representative analysis of the used extracted resin are depicted in Supplemental Figure 1A (^1H -NMR) and Supplemental Figure 1B (^{13}C -NMR). We did not observe any characteristic aromatic C-H signals (^1H -NMR; 7–8 ppm chemical shift range) or aromatic carbon signals (^{13}C -NMR 100 – 140 ppm range), providing no evidence for the accumulation of phenolic compounds (*i.e.* meta-cresol) on the column resin. However, the data clearly illustrated the presence of aliphatic organic compound(s); these proton signals were not observed in the extracted unused resin samples (data not shown). Therefore, these NMR data illustrate that degraded proprietary end-cap material could be observed in material extracted from used columns. Furthermore, when the used resin extracts were analyzed by GC/MS, we observed evidence for a variety of small molecular weight compounds (Figure 2, blue trace), but meta-cresol (the preservative in the insulin lispro drug product) was not observed. Hence, these data do not support the notion that phenolic compounds are accumulating on the column and causing additional non-specific hydrophobic interactions between the insulin analyte and the column surface that could explain the change in chromatographic signal.

We next treated new (unused resin) with meta-cresol (present in the lispro buffer) or zinc chloride at concentrations consistent with the insulin lispro drug product. As shown in Figure 2, we present GC/MS chromatograms from extracted samples obtained from new resin treated with i) water only; ii) meta-cresol (in the insulin lispro formulation buffer); iii) ZnCl_2 in water; and iv) used resin. Compared to used resin, unused extracted resin (Figure 2, water treated control, black trace) displayed evidence for only a few volatile components, whereas the meta-cresol treated resin clearly presented meta-cresol at $t_R \sim 11$ min. When the new resin was treated with ZnCl_2 , we observed a variety of volatile organic components also observed from extracted used resin. However, as compared to the used resin samples, the ZnCl_2 treated resin samples displayed much lower concentrations. We attribute the differences to two major factors: i) we did not control for zinc exposure in the used resin samples, the used column material had been exposed to far more zinc ions than our new resin zinc ion doping experiments due to the many column injections in the previous stability study; and ii) the used columns were stored for a long period of time (greater than 12 months) after being initially used; hence, it is likely that additional degradation events occurred over time.

At this point, the data appeared consistent with the notion that zinc ions were the primary culprit causing the SEC performance issues. If correct, then the addition of a chelator (*e.g.* EDTA) to the mobile phase should help reduce the rate of column degradation. To test this, we treated new resin with ZnCl_2 and EDTA, using a three-fold molar excess of EDTA relative to ZnCl_2 . We then repeated the GC/MS analysis (Figure 3) and while the resin

leached a number of species when treated with water, two species observed at ~17.3 and ~18.6 min were significantly decreased when EDTA was included. These results confirm that the column degradation was due, at least in part, to end-cap degradation facilitated by zinc ions. From these observations, we asked the final question: by adding EDTA to the mobile phase, can we maintain better and extended SEC column performance? To address this, we used new columns and compared SEC analysis performed in the presence (Figure 4A) and absence (Figure 4B) of EDTA. When EDTA was present, we observed limited modification to the insulin peak over 240 injections (192 insulin lispro, 48 BSAU); the BSAU standard was completely unaffected (data not shown). However, when we switched to the mobile phase without EDTA, we observed a steady growth in the post-main peak shoulder indicating column degradation. These data are further evidence that the released zinc ions are catalyzing column degradation.

Conclusion

Our experiments demonstrate that: i) zinc ions are a significant contributor to the observed SEC column degradation when performing insulin sample analysis; and ii) the addition of EDTA, a zinc ion chelating agent, to the mobile phase markedly helped to maintain column performance. At least for zinc-containing insulin samples using these SEC columns, we recommend the addition of EDTA to the mobile phase in order to protect the column and help ensure artifacts – due to column degradation events – are not misinterpreted as physiochemical changes in the insulin analyte. These findings also present the possibility that other metals commonly incorporated into biopharmaceutical production, such as tungsten from staked needles in prefilled syringes or copper which may be utilized in cell culture methods, may also possibly contribute to poor analytical SEC column performance. Therefore, we encourage others to investigate metal-catalyzed column surface degradation as a possible issue when developing robust SEC methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

BMT thanks Dr. Nicole Payton for her help and advice regarding GC/MS usage. We would also like to thank our reviewers for their insightful and helpful comments. This research utilized services of the Medicinal Chemistry Core Facility (MCCF) housed within the Department of Pharmaceutical Sciences (DOPS) at the University of Colorado Anschutz Medical Campus. The MCCF receives funding via Colorado Clinical and Translational Sciences Institute grant NIH-NCATS, UL1TR001082.

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Figure 1.

Sixteen insulin lispro drug product peaks eluted from a Tosoh G2000SWXL column during a 128-injection stability study by Teska et al (2014). Injections are colored from blue to red in order of increasing injection number. Peak position shifted to longer retention times with a post-main peak shoulder becoming very apparent in later injections (24–27 min). The main insulin peak elutes with a weight average-molecular weight of 14.2 ± 0.3 kDa.

Figure 2.
GC-MS chromatogram overlays: Extracted used resin (blue); new resin treated with ZnCl₂ (green); new resin treated with insulin lispro buffer containing meta-cresol (red); and new resin treated with MilliQ water (black). The peak eluting at 11.4 min (red) is meta-cresol.

Figure 3.
GC-MS chromatogram overlay: Water treated (green), ZnCl₂ treated (blue), and ZnCl₂ + EDTA (red) treated new resin samples. Species eluting at approximately 17.3 and 18.6 min (black arrows) were significantly lower in EDTA treatment group.

Figure 4. SEC chromatograms of 192 injections of insulin lispro eluted with EDTA (440 μ M) in the MP (A) and 192 injections of insulin lispro eluted without EDTA in the MP (B). Injections are colored from blue to red with increasing injection number. Insulin lispro elutes between 20–35 min at a weight-average molecular weight of 14.2 ± 0.3 kDa, and meta-cresol elutes between 35–50 min.