NOTE

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

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Received 5 September 2014; revised 27 October 2014; accepted 4 December 2014

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24330

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 lifespan. In these studies, we used multiangle light scattering, nuclear magnetic resonance, and gas chromatography–mass spectrometry methods to evaluate column degradation. The results from these studies illustrate: (1) that zinc ions introduced by the insulin product produced the observed column performance issues; and (2) that including ethylenediaminetetraacetic acid, a zinc chelator, in the mobile phase helped to maintain column performance. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: chromatography; mass spectrometry; chelation; NMR spectroscopy; surface chemistry

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.[1] observed a rapid decrease in the SEC column performance; in fact, it only required a moderate number of sample injections (~80–100) to produce this observation. 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.

As many diabetics require multiple injections per day in order to maintain an acceptable blood glucose level, marketed insulin and insulin analogs are formulated in multidose vials.[4] Furthermore, multiple needle insertions into a sterile drug product vial inherently increase the possibility of bacterial contamination. The US FDA (Food and Drug Administration) requires drug products in multidose vials to include an antimicrobial preservative. In the case of insulin formulations, phenol and/or metacresol are commonly employed. Insulin is somewhat of a special case, as it is well established that the presence of phenol and/or metacresol promotes favorable conformational changes in the insulin 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[1,10–13] 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—because of the concentration dependence of insulin self-association[15]—that the dilution into the column's flowing mobile phase (MP) intrinsically triggers the insulin hexamers to dissociate and ultimately release bound zinc ions (Zn^{+2}) and the various phenolic additives. On the basis of observations by Teska et al.,[1] we hypothesized two fundamental explanations for the observed SEC column performance issues: (1) either the phenolic preservatives were accumulating on the silica, creating a more hydrophobic surface; and/or, (2) 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 MPs, formulations, and buffers was purified through a Millipore Synergy UV (Millipore, Billerica, Massachusetts) filtration unit (MilliQ). Deionized water was used to rinse the liquid–liquid extraction sample vials.

Silica Resin Incubation

Tosoh G2000SWXL top-off resin (2.0 g; Tosoh Corporation, King of Prussia, Pennsylvania) 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 resuspended by gentle shaking. For each sample, this process was

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This article contains supplementary material available from the authors upon request or via the Internet at http://wileylibrary.com.

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 ethylenediaminetetraacetic acid (EDTA) in water; or a 14 mM Na_3PO_4, 174 mM glycerol, 30 mM metacresol in water (referred to as lispro buffer) was added. The tubes were capped and shaken to resuspend the silica resin. Samples were incubated (18°C–22°C; 24 h) 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 that could be easily pipetted (~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 (~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 gas chromatography–mass spectrometry (GC–MS), or diluted in $CDCl_3$ (1.0 mL) and analyzed by nuclear magnetic resonance (NMR).

Size-Exclusion Chromatography

Size-exclusion chromatography was performed on an Agilent 1100/1200 HPLC (Agilent Technologies, Santa Clara, California) detecting UV absorbance (280 nm). Multiangle light scattering (MALS) measurements were made with a Wyatt DAWN EOS 18-angle MALS detector (Wyatt Technology Corporation, Santa Barbra, California) plumbed in series with the UV detector. Molecular weight calculations were carried out in Wyatt's ASTRA software (version 5.3.4). The MP 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 x 300 mm^2; Tosoh Corporation) 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 EDTA (440 μM) as the 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, Illinois) and used as our SEC reference standard, and referred to as BSAU. The injection sequence used was as follows: BSAU, one injection; insulin lispro, eight injections; BSAU, one injection. This sequence was repeated (24 times) for each MP condition (with and without 440 μm EDTA) resulting in a total of 192 insulin lispro injections and 48 BSAU injections per MP condition. Samples were stored in amber HPLC vials in the autosampler at room temperature (18°C–22°C) over the 9-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.

NMR and GC–MS

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 tetramethylsilane ($\delta = 0.0$) as an internal standard. GC–MS was performed on a Shimadzu GC/MS-QP2010 Plus gas chromatograph mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, Maryland). The GC2010 was equipped with an Rtx-5MS column (0.25 μm thickness, 30.0 m, 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 to 750 *m/z* from 7.0 to 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/s, 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. As 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.[18–20] 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 MP in order to assist in shielding the analyte from the charged uncapped groups on the surface of the silica.[21–25] 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 MP 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 ± 1°C and injected once for every eight experimental insulin sample injections. After approximately 80 total injections, column performance issues were apparent (Fig. 1; yellow traces) and clearly visible after approximately 100 insulin injections (Fig. 1, red traces).[1]

DOI 10.1002/jps.24330