Effects of Phenol and meta-Cresol Depletion on Insulin Analog Stability at Physiological Temperature

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ABSTRACT: The stability of three commercial "fast-acting" insulin analogs, insulin lispro, insulin aspart, and insulin glulisine, was studied at various concentrations of phenolic preservatives (phenol and/or meta-cresol) during 9 days of incubation at 37°C. The analysis by both size-exclusion and reversed-phase chromatography showed degradation of lispro and aspart that was inversely dependent on the concentration of phenolic preservatives. Insulin glulisine was much more stable than the other analogs and showed minimal degradation even in the absence of phenolic preservatives. With sedimentation velocity ultracentrifugation, we determined the preservatives' effect on the insulins' self-assembly. When depleted of preservatives, insulin glulisine dissociates from higher molecular weight species into a number of intermediate molecular weight species, in between monomer and hexamer, whereas insulin aspart and insulin lispro dissociate into monomers and dimers. Decreased stability of insulin lispro and insulin aspart seems to be because of the extent of dissociation when depleted of preservative. Insulin glulisine's dissociation to intermediate molecular weight species appears to help minimize its degradation during incubation at 37°C. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:2255–2267, 2014
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INTRODUCTION

Endogenous human insulin monomers assemble into dimers, which further assemble into hexamers coordinated by two zinc ions.[1] Both hexamers and dimers are biologically inactive in the context of regulating the uptake of glucose. However, they are much more conformationally stable than the insulin monomer,[2] resulting in greatly reduced aggregation, fibrillation, and chemical degradation during storage compared with the monomer.[3] The hexamer is further stabilized by binding phenolic compounds, such as phenol and meta-cresol (m-cresol) to a hydrophobic pocket in the monomer–monomer interface within the hexamer. This induces a structural change in the B-chain residues B1–B8 from an open loop (T-state) to an α-helix (R-state),[4,5] which has been shown to have increased stability.[6–10] The overall hexamer structure is designated as one of the three states depending on the number of monomers in the T- or R-state: T_6, T_3R_3, or R_6. In addition, the transition from the T- to the R-state is favored by heterotropic cooperativity with the anion-binding sites on the zinc ligand within the core of the hexamer.[11–13] This ligand-induced structural transition in the formation of the hexamer and the requisite process of dissociation to active insulin monomer delay the onset of biological action of human insulin,[14] but help reduce degradation of the protein in drug product formulations.[15–17]

Today, the vast majority of patients on insulin therapy use "fast-acting" recombinant human insulin analogs.[18] Point mutations in these analogs, designed specifically to disrupt the stabilizing intramolecular interactions of insulin dimers and hexamers, provide faster onset of biological action when compared with human insulin.[14,19,20] The three most common analogs used today are insulin lispro (Humalog™; Eli Lilly, Indianapolis, Indiana), insulin aspart (Novolog™; Novartis, Basel, Switzerland), and insulin glulisine (Apidra™; Sanofi Aventis, Bridgewater, New Jersey). Insulin lispro is modified by reversing the penultimate two amino acids in the natural human insulin B-chain (from Pro^{B28}-Lys^{B29} to Lys^{B28}-Pro^{B29}), which weakens backbone hydrogen bonding between monomers.[21,22] As with human insulin, lispro forms stable hexamers in the presence of zinc and phenol.[23] However, the weakening of monomer–monomer interactions in the dimer allows for quicker dissociation from hexamer to the biologically active monomer, resulting in an earlier onset and shorter duration of action than human insulin.[24,25] Lispro has been shown to be mostly hexameric in the pharmaceutical formulation that contains both zinc and phenol.[26] In the one published study on aspart and lispro pharmaceutical stability, Lougheed et al.[27] found des-amido degradation products of lispro in infusions systems.

In insulin aspart, the proline at position 28 on the B-chain is replaced with a charged aspartic acid residue. The removal of the B28 proline weakens monomer–monomer backbone hydrogen bonding[8] in much the same way that lispro self-association is destabilized. This decreases aspart's propensity to dimerize, when compared with human insulin,[15] and leads to quicker

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onset and shortened duration of action.[24] Jars et al.[28] have shown that aspart forms predominantly Asp/IsoAsp degradation products at positions B3 (like lispro) and B28.

Insulin glulisine has two substitutions: asparagine to lysine at position B3 and lysine to glutamic acid at position B29. Unlike aspart or lispro dimers, both of which are primarily weakened by the removal of the B28 proline, insulin glulisine leaves the B28 proline intact and instead uses charge–charge repulsion between monomers to destabilize the hexamer.[29] Glulisine is formulated without zinc, and it is expected that glulisine in the drug product does not significantly self-associate into hexamers.[29] However, to our knowledge, there are no published reports on glulisine self-association, structure, or stability in pharmaceutical formulations. However, recent work has shown that degradation by fibrillation to be comparable to that of lispro.[30]

Therapeutic insulin analogs are often delivered by subcutaneous injection. With an average patient needing around three injections per day,[31] continuous insulin infusion pumps, which were introduced in the early 1980s, can provide a more convenient mode of treatment. Wearable, "online" blood glucose monitors[32–34] are being incorporated into insulin pumps and, over the long term, it is expected that the enhanced control with these combined technologies will become the preferred mode of therapy for a large proportion of patients.

Since the insulin pump was introduced, however, there have been numerous problems with insulin stability under the conditions within the pumps. For example, insulin has been found to be aggregated in the pump reservoir and tubing[35] and in some cases to have precipitated.[36–38] These two degradation pathways are suspected to lead to catheter occlusion—a continuing clinical problem with analogs in insulin pumps,[24,39–48] which may occur in more than half of catheters using fast-acting insulin analogs longer than 48 hours.[49]

A number of studies also have shown that levels of phenolic compounds, which are included in pharmaceutical formulations as antimicrobial preservatives as well as insulin stabilizers, are depleted by absorption into the insulin pump catheters,[35,50,51] with as little as 10% remaining after 24 h.[35] Experiments performed in our laboratory with currently available catheter sets showed the complete loss of m-cresol upon incubation at 37°C for 48 h.[52] Because phenolic preservatives play such important roles in human insulin self-assembly, structure, and storage stability,[8,30] we hypothesized that depletion of these stabilizing ligands would decrease the extent of hexamer assembly in these insulin analogs as well. Subsequently, with more insulin analog molecules in the monomeric state, we expect reduced stability at physiological temperature. To test this hypothesis, we removed preservatives from marketed insulin analog formulations, leaving the remainder of the formulation intact. We then analyzed the assembly state of each of the analogs with and without phenolic preservatives by analytical ultracentrifugation (AUC). Next, we added various fractions of the original amount of phenolic preservatives back into the formulation and incubated samples quiescently at 37°C for 9 days. At various time points throughout the incubation, samples were analyzed for physical degradation by size-exclusion chromatography (SEC) and chemical degradation by reversed-phase (RP) chromatography to determine the effect of reduced phenolic preservative levels on stability.

MATERIALS AND METHODS

Materials

Insulin analogs were purchased as commercial drug products from a local pharmacy and were used before their expiry dates. All insulin analogs were purchased in 10 mL vials. Insulin lispro is formulated in a 1.88-mg/mL sodium phosphate buffer with 16 mg/mL glycerol, 3.15 mg/mL m-cresol, and 0.0197 mg/mL zinc.[53] Insulin aspart is formulated in a 1.25-mg/mL sodium phosphate buffer, with 16 mg/mL glycerol, 0.58 mg/mL sodium chloride, and 0.0196 mg/mL zinc, and uses a combination of 1.5 mg/mL phenol and 1.72 mg/mL m-cresol as antimicrobial agents.[54] Insulin glulisine is formulated in 6 mg/mL tromethane buffer, 5 mg/mL sodium chloride, 3.15 mg/mL m-cresol, and 0.01 mg/mL Tween 20.[55]

All laboratory chemicals used were of analytical grade or higher and were used without further purification. Water used in mobile phases, formulations, and buffers was purified through a Millipore Synergy UV (Billerica, Massachusetts) filtration unit.

Stability Study

Phenol and m-cresol were removed from insulin analogs using Zeba (Thermo Scientific, Rockford, Illinois) desalting columns. The desalting columns were washed three times with formulation buffer not containing phenolic preservatives, and analogs were eluted from the desalting column using the same buffer, as has been performed previously.[30] A significant number of particles were shed from the Zeba columns during preparation. These particles were removed by preparative ultracentrifugation at approximately 110,000*g* for 70 min at 4°C. The supernatant from the ultracentrifugation step was concentrated to approximately 8 mg/mL using Amicon Ultra 3000 MWCO centrifugal filters (Millipore, Cork, Ireland) and then diluted to 6.94 mg/mL (two times the marketed insulin concentration). The concentrated insulin analog solutions were then diluted to a final concentration of 3.47 mg/mL and final phenol and/or m-cresol concentrations of 0%, 20%, 40%, 60%, 80%, and 100% of those in each of the respective insulin analog commercial formulation (Table 1). Two-hundred microliter samples were pipetted into 1.3 mL glass lyophilization vials (West Pharmaceuticals, Exton, Pennsylvania). Vials were capped and incubated quiescently at 37°C. Three separate vials for each sample type were tested at each of the 0-, 1-, 6-, and 9-day time points.

Size-Exclusion Chromatography

None of the insulin analogs we studied have any tryptophan residues. Therefore, the strongest UV absorbance comes from tyrosine residues that unfortunately have an absorbance spectrum that overlaps those of m-cresol and phenol, which are present in nearly 50-fold molar excess in the marketed formulations. In addition, HPLC SEC column performance degrades rapidly with these samples because of insulin aggregates, phenolic preservative, and/or zinc interactions with the column.[52,56] To overcome these limitations and avoid the pitfalls of the US Pharmacopoeia SEC method for insulin,[57] we developed a UPLC-SEC method. Insulin monomer and soluble aggregates were detected and quantified by SEC using Waters Acquity UPLC BEH200 (Waters Corp., Milford, Massachusetts) SEC column (4.6 × 150 mm^2) on an Agilent 1100/1200 HPLC (Agilent Technologies, Santa Clara, California). To remove large

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