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Comparison of the Purity and Impurity of Glucagon-for-Injection Products under Various Stability Conditions

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Abstract: Glucagon is a polypeptide hormone that serves as an essential therapeutic agent in the emergency treatment of hypoglycemia. Recently, the first generic glucagon for injection was approved. However, unlike its brand name counterpart, which is produced via recombinant DNA, the generic glucagon is produced using a chemical synthesis method. Regardless of its origin, impurities may occur in both glucagon drug products. While these impurities may greatly compromise the safety and efficacy of the glucagon drug products, studies accessing the impurities of glucagon for injection are limited. This manuscript analyzed the stability and impurities of a generic and brand glucagon for injection, including desamido and non-desamido impurities, under various storage and temperature conditions using an ultra-performance liquid chromatography method. The glucagon products were analyzed after 6 and 24 months of storage under room temperatures (20–25 °C). In addition, the products were also assessed after 6 months of storage under high temperatures (40 °C). Under each stability storage condition, three lots of the synthetic glucagon were evaluated by UPLC with at least one lot of the recombinant glucagon for comparison. A total of 37 peaks were identified (except for the solvent peaks, which appeared at retention times less than 1.5 min) from the synthetic and recombinant glucagon lots. It was found that the number of impurities observed in the synthetic glucagon were lower than the referenced recombinant glucagon across all stability conditions. Throughout all tested conditions, the synthetic glucagon for injection had an averaged purity of 92.8–99.3%, while the referenced recombinant drug had an averaged purity of 70.3–91.7%. Based on the study results, it can be concluded that the impurity profile for the synthetic glucagon for injection has a comparable and even lower level of impurities than the recombinant version under all stability conditions.

Keywords: glucagon; HPLC; impurity; stability; synthetic peptide; recombinant; injection

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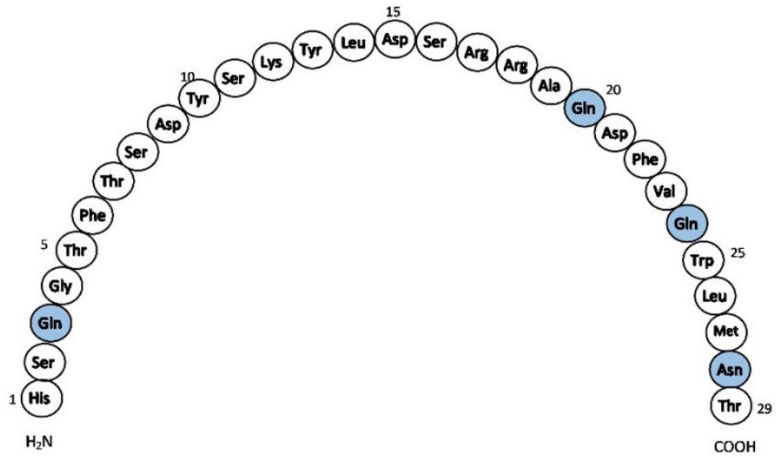
1. Introduction

Glucagon is a 29-amino acid polypeptide hormone whose major function is to promote glucose levels in the bloodstream [1]. Due to this, glucagon serves as an essential therapeutic agent in the emergency treatment of hypoglycemia [1]. However, glucagon is inherently unstable and it has been shown to degrade in aqueous solutions [2]. To maintain its stability, pharmaceutical preparations of glucagon such as GlucaGen® HypoKit® (Novo Nordisk, Princeton, NJ, USA) or Glucagon™ (Eli Lilly and Company, Indianapolis, IN, USA) are formulated as lyophilized powder to be reconstituted with a diluent for immediate use [3]. Although both drug products have been approved and used on the market for over 20 years, there had been no generic version of either product until recently [4].

The first generic equivalent to Eli Lilly's glucagon-for-injection kit was approved by the FDA on December 2020, which demonstrated sameness in terms of its safety and efficacy to its brand name counterparts [4]. Similar to its brand name version (i.e., Eli Lilly's Glucagon™), the generic glucagon is supplied as lyophilized powder to be reconstituted with a diluent for immediate use. However, a key difference between the generic and

brand version is that the generic glucagon is manufactured using a chemical synthesis method (synthetic) while the brand glucagon is of recombinant DNA (rDNA) origin [5]. In short, recombinant peptides are produced through molecular cloning techniques using host vectors containing the inserted DNA fragment of interest (e.g., glucagon, insulin, etc.). The inserted DNA fragment is then reproduced and extracted [6]. On the other hand, the generic glucagon is chemically synthesized using a solid-phase peptide synthesis (SPPS) method. SPPS involves step-wise additions of protected amino acid derivatives to a growing peptide chain, followed by deprotection and washing steps to remove unreacted groups and also side products [7]. A notable advantage of synthetic peptides is their ability to generate copies of the fragments of interest through diverse chemical modifications.

Regardless of how the glucagon is produced, the drug product may contain impurities as a result of degradation, due to storage temperature or duration. A common degradation pathway is deamidation, which involves hydrolysis of its Asn and Gln side chain amide groups [8]. Past studies on the chemical instability of glucagon showed that the major hydrolytic degradation pathways of glucagon include deamidation at Gln-3, Gln-20, Gln-24, and Asn-28 [9–11], as shown in Figure 1. The modification of amino acid side chains such as deamidation may induce unwanted immune responses (immunogenicity) [12,13], which may compromise the safety and efficacy of the drug product [14,15]. Therefore, the purity and stability of drug products are very critical in minimizing the risk of immunogenicity.



NH_2-His[1]-Ser[2]-Gln[3]-Gly[4]-Thr[5]-Phe[6]-Thr[7]-Ser[8]-Asp[9]-Tyr[10]-Ser[11]-Lys[12]-Tyr[13]-Leu[14]-Asp[15]-Ser[16]-Arg[17]-Arg[18]-Ala[19]-Gln[20]-Asp[21]-Phe[22]-Val[23]-Gln[24]-Trp[25]-Leu[26]-Met[27]-Asn[28]-Thr[29]-COOH

Figure 1. Amino acid structure of glucagon. Blue shaded sites are susceptible to deamidation modification.

An impurity and stability study of glucagon for injection by ultra-performance liquid chromatography (UPLC) has not been reported before. This manuscript is the first to analyze the stability and impurity of glucagon, including desamido and non-desamido impurities, under various storage and temperature conditions using an UPLC method.

2. Materials and Methods

2.1. Sample Preparation

Three lots of the generic (synthetic) glucagon for injection were provided by Amphastar Pharmaceuticals, Inc. (Rancho Cucamonga, CA, USA). The branded glucagon for injection (Eli Lilly and Company's Glucagon™, Indianapolis, IN, USA) was purchased from the U.S. market and used as a reference-listed drug for comparison. In this study, the synthetic glucagon will be referred to as (AMP-glucagon) and the recombinant glucagon will be referred to as (ELI-glucagon).

To evaluate the impurity and stability profile of glucagon, AMP-glucagon and ELI-glucagon were analyzed after 6 and 24 months of storage under room temperatures

(20–25 °C). In addition, the products were also assessed after 6 months of storage under high temperatures (40 °C). Under each stability storage condition, the three lots of AMP-glucagon would be evaluated with at least one lot of ELI-glucagon for comparison. The different storage times and temperatures, as well as the age of samples at testing are indicated in Table 1.

Table 1. Summary of the synthetic and recombinant glucagon samples tested.

Storage Condition		AMP-Glucagon		ELI-Glucagon	
		Lot #	Age at Testing	Lot #	Age at Testing
Initial	-	021914 021914A 021914B	0	C464145A *	10
6 Months	25 °C 40 °C	021914 021914A 021914B	6	C215547C **	13
24 Months	25 °C †	021914 021914A 021914B	24	C304844A *** C304844C ***	24

* Newly purchased and tested immediately. ** placed on stability together with AMP-glucagon and tested after 6 months. *** tested with AMP-glucagon assuming a 24 month shelf-life for RLD. † 20–25 °C for ELI-Glucagon.

For the initial or time zero test, the ELI-glucagon units were purchased commercially. At the time of purchase, it is likely that several months had passed since the time of manufacture. Therefore, some initial variability was expected. For the 6 month stability test, the newly purchased ELI-glucagon samples were placed on stability chamber with AMP-glucagon for 6 months before testing. For the 24 month impurity test, ELI-glucagon samples were stored at room temperature and tested when the units expired. It was assumed that ELI-glucagon has 24-months of shelf-life.

2.2. UPLC Method

A reversed-phase UPLC method was developed based on the U.S. Pharmacopeia (USP). The Acquity UPLC systems (Waters, Milford, MA, USA), equipped with a photodiode array detector, was employed for this study. The separation of the glucagon drug from the impurities was carried out using a C18 (1.7 µm, 2.1 mm × 100 mm) column (Waters Acquity BEH300) at 45 °C with a mobile phase flowing rate of 0.4 mL/min. Mobile phase A (phosphate buffer) and mobile phase B (acetonitrile) were used for the elution of the components during the 30 min run time. The buffer solution, mobile phase A, was prepared by dissolving 16.3 g monobasic potassium phosphate in 750 mL water, adjusted to pH 2.7 with phosphoric acid. Mobile phase B was prepared by mixing 400 mL acetonitrile with 600 mL of water. A standard solution was prepared by reconstituting a vial of USP recombinant glucagon reference standard with 6 mL of diluents. The injection volume and wavelength were fixed at 4 µL and 214 nm, respectively. The UPLC system conditions as well as the mobile phase elution program are detailed in Table 2. Each glucagon sample was reconstituted with 2 mL of diluents and tested immediately.

The UPLC method was validated with respect to linearity, accuracy, precision (repeatability and intermediate precision), quantitation limit, stability of solutions, and specificity (including selectivity and stress studies), following ICH guideline Q2 (R1) [16]. The identified related substance (four desamido glucagon) was separated from the glucagon peak. All the detected peaks were quantitatively evaluated. The quantitative limit (QL) for this UPLC method is 0.15% of the total peak area, and its detection limit (D.L.) is 0.05% of the total peak area. The equivalency between the UPLC method and USP method was evaluated and the UPLC method used in this study was found to be equivalent or more efficient than the USP method.

Table 2. UPLC system conditions.

Item	Description
Instrumentation	Waters Acquity UPLC system equipped with PDA detector
Column	Waters Acquity UPLC BEH300 C18, 1.7 µm, 2.1 mm × 100 mm, PN 186003686
Mobile Phase Composition	A: 65%; B: 35%
Flow Rate	0.4 mL/min
Column Temperature	45 °C
Injection volume	4 µL
Run time	30 min
Detector Wavelength	214 nm
Sample Temperature	6 °C
Weak Wash (0.1% H_3PO_4 in 10% ACN)	1200 µL
Strong Wash (MPB)	400 µL

2.3. Data Analysis

This study used the USP specification for impurities as the standard criteria for analysis, in which the total desamido impurities and total impurities in glucagon for injection should not be more than 14 and 31%, respectively [17]. Further, according to the regulatory guidance for peptide drugs such as glucagon, any individual impurities detected should not be greater than 0.5% [18]. Therefore, in this study, the peak area percentage for (i) purity, (ii) total impurities detected, (iii) total impurities with the peak area >0.5%, (iv) highest single peak area percentage, (v) peak area percentage of all desamido impurities, and (vi) peak area percentage of non-desamido impurities at each storage condition were evaluated, with the mean and standard deviation provided.

2.4. Equivalence Evaluation Criteria

To evaluate whether the impurity percentage meets the standard acceptance criteria, the equivalence evaluation criteria (EEC) was established. If the studied impurity is listed in the USP specification with a peak area percentage, the USP was used as the upper limit for the equivalence evaluation. For glucagon for injection, total desamido impurities and total impurities are specified in the USP monograph to be no more than 14 and 31%, respectively. For items without a USP specification, equivalence was established by evaluating the purity of the product along with its individual impurities or group of impurities. For the evaluation of purity, the EEC will have a lower limit based on the following formula:

$$\text{Purity EEC (lower limit)} = R_{Min}^{(p)} \times (1 - \theta) \quad (1)$$

where $R_{Min}^{(p)}$ is the observed minimum purity peak area percentage for the glucagon peak in the RLD samples at any given time at room temperature (~20–25 °C), and θ is the allowance percentage, proposed as 5%.

For the evaluation of individual impurities or impurity groups, the EEC will have an upper limit only, with no lower limit. The EEC upper limits were proposed as follows:

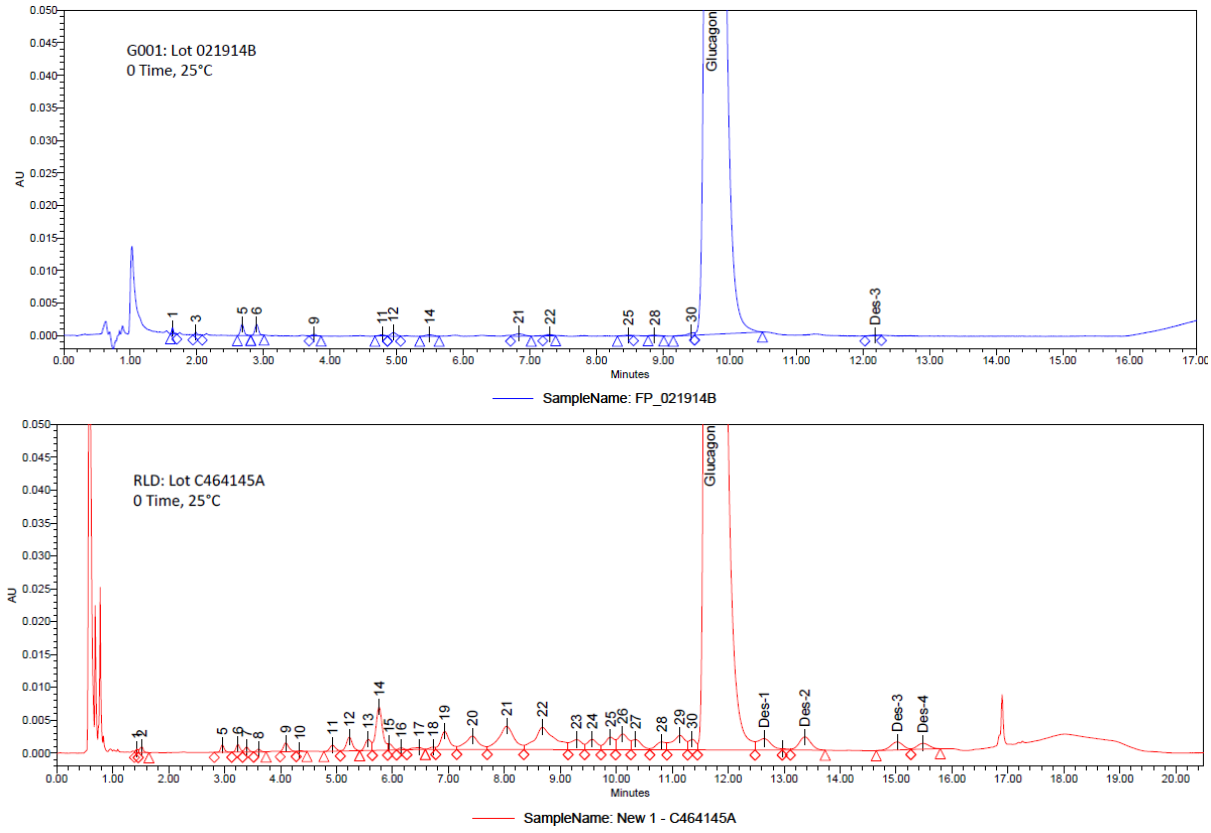
$$\text{Impurity EEC (upper limit)} = Max\left[R_j \times (1 + \eta), \left(R_j + \delta\right)\right] \quad (2)$$

where R_j is the corresponding impurity amount observed in the referenced ELI-glucagon for jth impurity peak (i) at the same time and the similar storage temperature or (ii) at the earlier time and the similar storage temperature. In the case where multiple lots of ELI-glucagon were tested, the maximum amount of the impurity found in the ELI-glucagon was used to determine the EEC upper limit. The percentage allowance (η) was proposed to be 25% and the quantitative limit (δ) was 0.15% of the total peak.

3. Results and Discussion

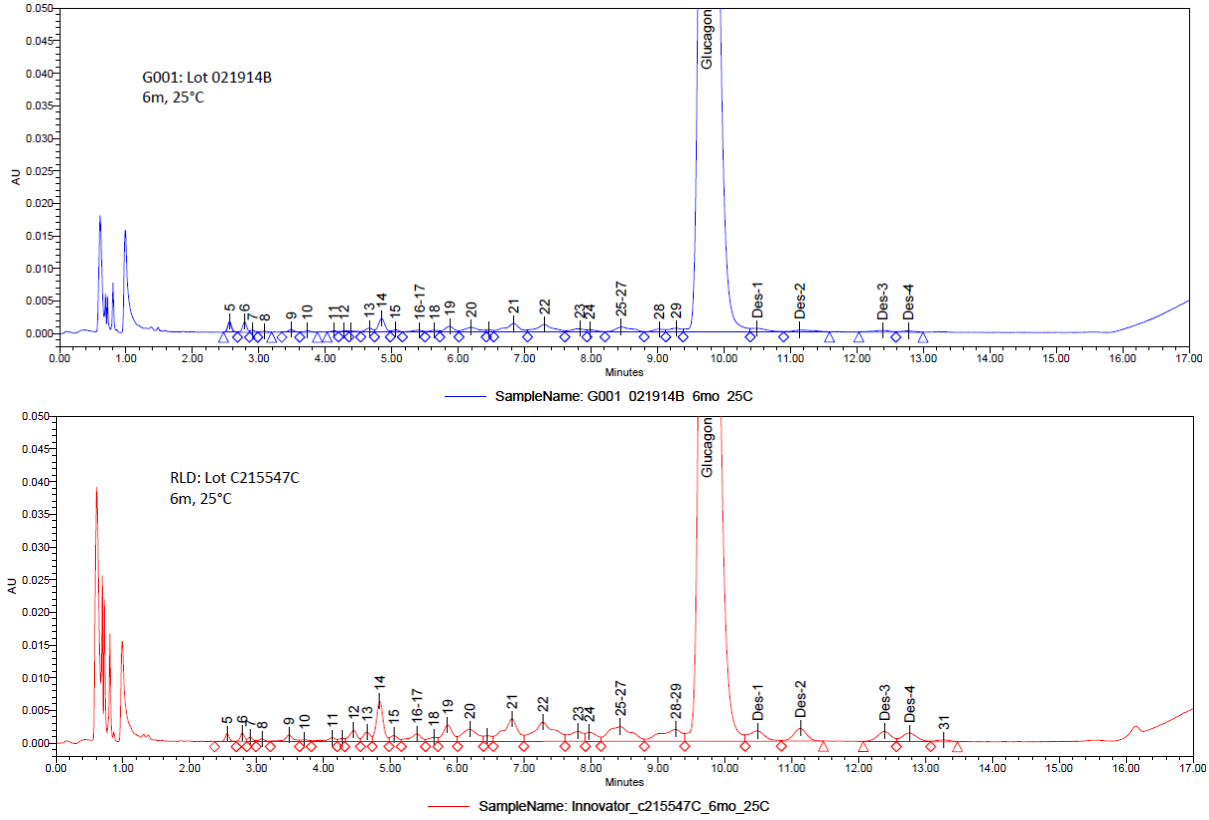
Different glucagon-for-injection samples under different storage conditions were analyzed by UPLC. A total of 37 peaks were identified (except for the solvent peaks, which appeared at retention times of less than 1.5 min) from the AMP-glucagon and ELI-glucagon lots. Peak 31 was identified as "glucagon" at approximate retention times between 9.13–11.94 min for the stability conditions tested. The four USP-specified desamido impurity peaks were detected, which appeared after the glucagon peak, and were marked as "Des-1", "Des-2", "Des-3", and "Des-4", respectively, on the chromatograms. Thirty-three peaks are unknown impurities, marked as Imp-1 to Imp-30, which appeared at an earlier retention time than the glucagon peak, and Imp-31 and Imp-32, which appeared at a later retention time. Imp-32 peak only appeared under the accelerated storage condition (6-month at 40 °C) at retention times of 14.45–17.73 min (glucagon relative retention time of 1.581–1.930). Representative UPLC chromatograms of AMP-glucagon and ELI-glucagon impurity peaks at zero time, 6 months at 25 °C, 24 months at 25 °C, and 6 months at 40 °C are provided in Figure 2A–D, respectively. Data for the retention times (RT, min) and the relative retention times for each peak for each sample lot are provided in the Supplementary Material.

The purity and impurity profiles of AMP-glucagon and ELI-glucagon were further quantitatively evaluated for purity (glucagon peak), each single impurity, the highest single impurity, total impurities, all desamido impurities, all impurities with a peak area >0.5%, and all other impurities. When a peak was observed but the peak area percentage was less than the detection limit (0.05%), it is recorded as "<D.L." in Table 3.

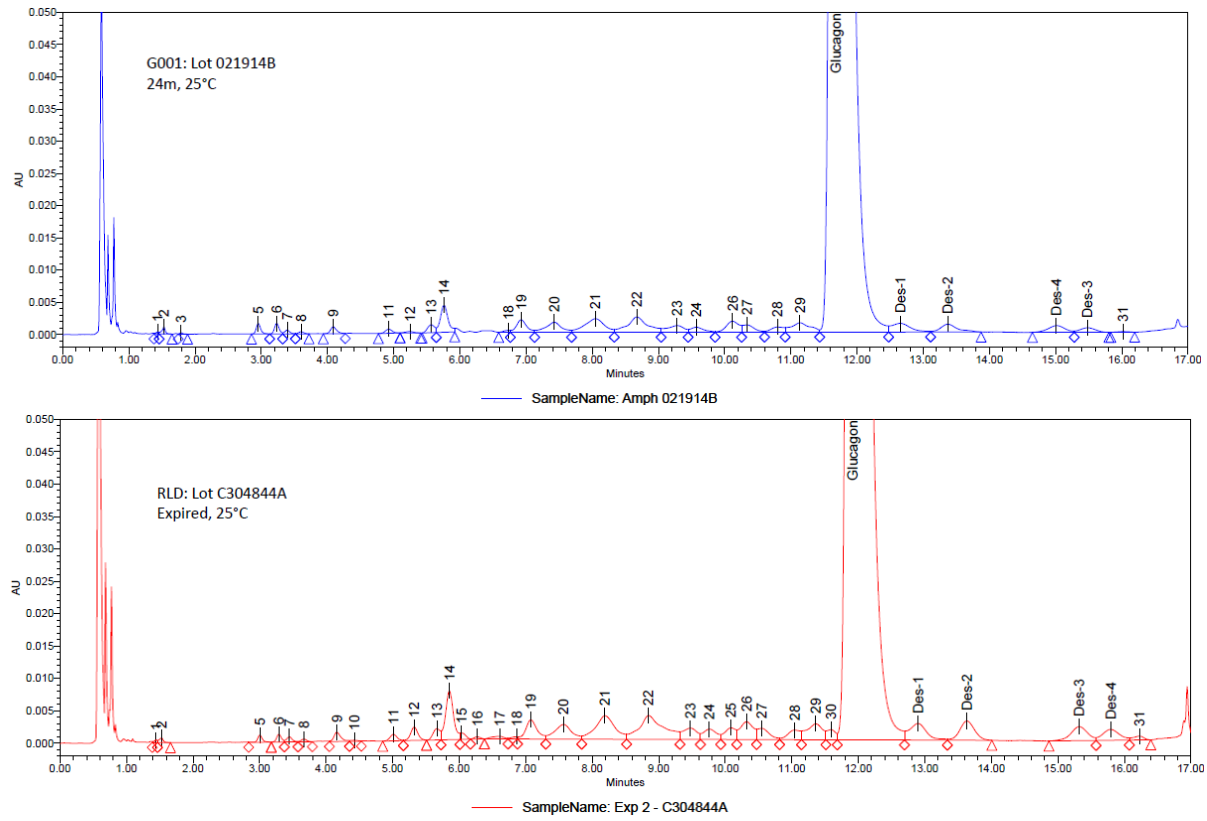


(**A**)

Figure 2. *Cont.*

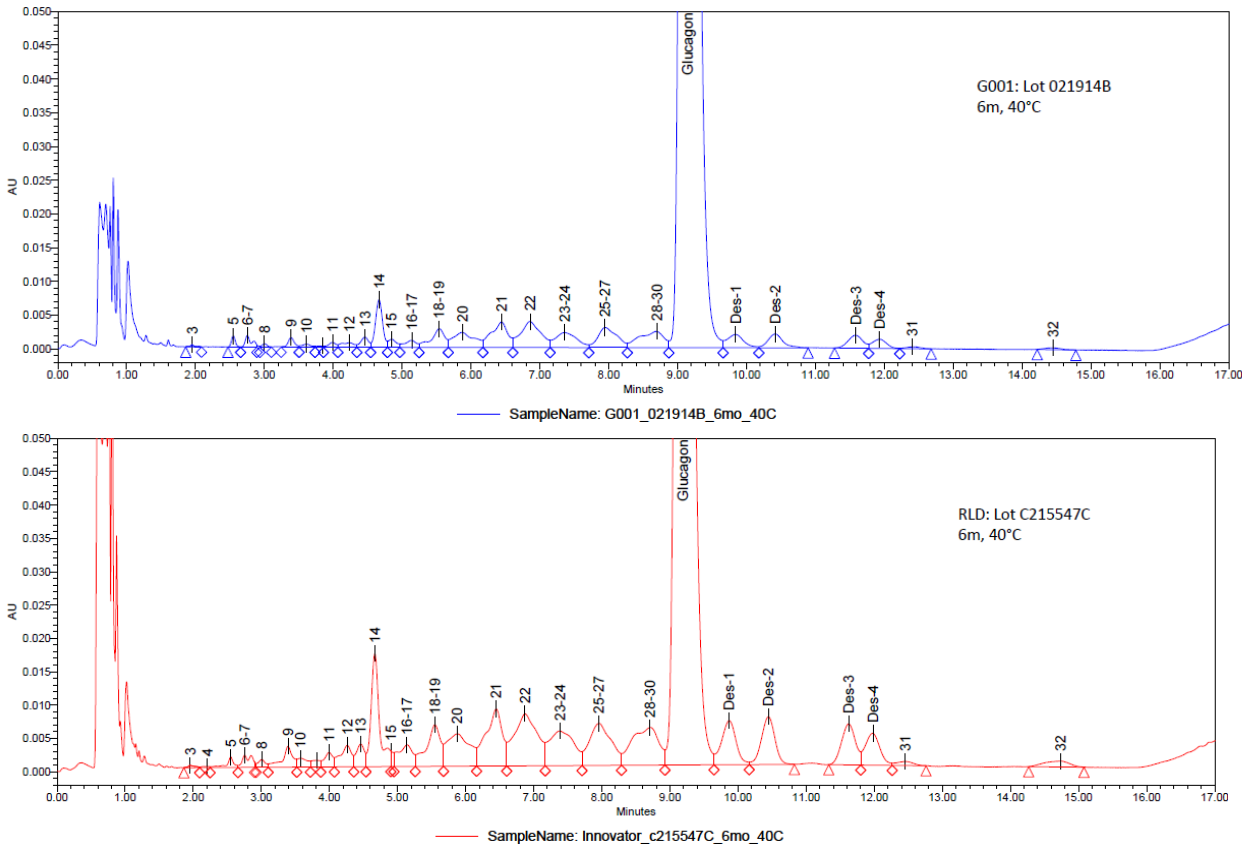


(**B**)



(**C**)

Figure 2. *Cont.*



(**D**)

Figure 2. G001 = AMP-glucagon; RLD = ELI-glucagon. (**A**). Representative chromatograms at zero time, 25 °C. (**B**). Representative chromatograms at 6 months, 25 °C. (**C**). Representative chromatograms at 24 months, 25 °C. (**D**). Representative chromatograms at 6 months, 40 °C.

It was found that the number of impurities observed (>D.L.) in AMP-glucagon were lower than the referenced ELI-glucagon across all stability conditions. ELI-glucagon, at the initial time (approximate age of 10 months), had 27 different impurities, with three impurities having peak areas of ≥0.5%. For AMP-glucagon, the number of impurities at the initial time (age of 0 month) were three to eight impurities among the three lots, and none had a peak area of ≥0.5% (Table 3).

After 6 months under 25 °C, the number of impurities above the detection limit had increased to 10–22 in AMP-glucagon, but all had a peak area percentage of below 0.5%. In comparison, the referenced ELI-glucagon had 26 impurities, of which nine had peak areas ≥0.5%.

At the end of shelf life or 24 months, the number of impurities in AMP-glucagon increased to 17–23 across the three lots, of which zero to three impurities had peak areas of ≥0.5%. Nonetheless, the number of impurities was still noticeably lower than the referenced expired ELI-glucagon, which had 27 impurities in both of the studied lots, with 9–10 impurities having peak areas of ≥0.5%.

For accelerated stability conditions, AMP-glucagon and ELI-glucagon were stored at extreme temperatures of 40 °C for 6 months. Under this condition, the three AMP-glucagon lots had impurities ranging from 14–23, with 0–11 impurities having peak areas of ≥0.5%. When compared to the referenced lot, ELI-glucagon again saw a higher number of 24 impurities detected, of which 15 had a peak area of ≥0.5% (Table 3).

Table 3. Peak area percentage of glucagon and impurities at 25 °C and 40 °C.

Peak	Peak Name	Initial Time, 25 °C					6 Months, 25 °C					24 Months, 25 °C						6 Months, 40 °C				
		AMP-Glucagon Lots			RLD *	EEC Upper Limit †	AMP-Glucagon Lots			RLD *	EEC Upper Limit †	AMP-Glucagon Lots			RLD*		EEC Upper Limit †	AMP-Glucagon Lots			RLD *	EEC Upper Limit †
		021914	021914A	021914B	C464 145A		021914	021914A	021914B	C215 547C		021914	021914A	021914B	C304 844A	C304 844C		021914	021914A	021914B	C215 547C	
1	Imp-1	<D.L.	<D.L.	<D.L.	<D.L.	0.15	-	-	-	-	0.15	<D.L.	-	<D.L.	<D.L.	<D.L.	0.15	-	-	-	-	0.15
2	Imp-2	<D.L.	<D.L.	-	0.05	0.20	-	-	-	-	0.20	0.07	0.05	0.06	<D.L.	<D.L.	0.20	-	-	-	-	0.20
3	Imp-3	<D.L.	<D.L.	<D.L.	-	0.15	-	-	-	-	0.15	<D.L.	<D.L.	<D.L.	-	-	0.15	<D.L.	-	<D.L.	<D.L.	0.15
4	Imp-4	-	-	-	-	0.15	-	-	-	-	0.15	-	-	-	-	<D.L.	0.15	-	-	-	<D.L.	0.15
5	Imp-5	0.14	0.13	0.12	0.07	0.22	0.15	0.14	0.16	0.12	0.27	0.14	0.12	0.11	0.07	0.08	0.27	0.15	0.15	0.12	0.17	0.32
6	Imp-6	0.13	0.14	0.12	0.08	0.23	0.17	0.16	0.17	0.13	0.28	0.15	0.12	0.12	0.08	0.10	0.28	0.21	0.19	0.19	0.27	0.42
7	Imp-7	-	-	-	0.06	0.21	<D.L.	-	<D.L.	0.07	0.22	<D.L.	<D.L.	0.05	0.06	0.08	0.23	-	-	-	-	0.23
8	Imp-8	-	-	-	<D.L.	0.15	-	-	<D.L.	0.06	0.21	<D.L.	-	<D.L.	<D.L.	<D.L.	0.21	<D.L.	<D.L.	<D.L.	0.13	0.28
9	Imp-9	<D.L.	<D.L.	<D.L.	0.12	0.27	<D.L.	<D.L.	0.06	0.20	0.35	0.09	<D.L.	0.11	0.14	0.16	0.35	0.09	<D.L.	0.13	0.52	0.67
10	Imp-10	-	-	-	<D.L.	0.15	<D.L.	-	<D.L.	0.05	0.20	-	-	-	<D.L.	<D.L.	0.20	0.05	<D.L.	0.05	0.35	0.50
11	Imp-11	<D.L.	-	<D.L.	0.09	0.24	<D.L.	-	<D.L.	0.14	0.29	0.06	<D.L.	0.07	0.10	0.12	0.29	0.05	-	0.09	0.30	0.45
12	Imp-12	0.05	-	0.06	0.21	0.36	0.05	<D.L.	0.06	0.35	0.50	<D.L.	-	<D.L.	0.22	0.22	0.50	0.10	<D.L.	0.13	0.58	0.73
13	Imp-13	-	-	-	0.17	0.32	0.08	<D.L.	0.10	0.22	0.37	0.11	<D.L.	0.14	0.17	0.20	0.37	0.15	0.06	0.19	0.45	0.60
14	Imp-14	0.06	<D.L.	<D.L.	0.71	0.89	0.24	0.08	0.33	0.90	1.13	0.36	0.11	0.50	0.86	0.98	1.23	0.57	0.22	0.88	2.28	2.85
15	Imp-15	-	-	-	0.07	0.22	<D.L.	-	0.05	0.15	0.30	<D.L.	-	-	0.08	0.10	0.30	0.10	<D.L.	0.16	0.10	0.30
16	Imp-16	-	-	-	<D.L.	0.15	0.06	0.05	0.07	0.29	0.44	<D.L.	-	-	<D.L.	<D.L.	0.44	0.16	0.06	0.19	0.73	0.91
17	Imp-17	-	-	-	0.05	0.20	-	-	-	-	0.20	0.08	0.05	-	0.07	0.07	0.22	-	-	-	-	0.22
18	Imp-18	-	-	-	<D.L.	0.15	<D.L.	<D.L.	0.06	0.12	0.27	<D.L.	-	<D.L.	<D.L.	<D.L.	0.27	0.44	<D.L.	0.63	1.41	1.76
19	Imp-19	<D.L.	-	-	0.39	0.54	0.11	<D.L.	0.19	0.53	0.68	0.22	0.07	0.31	0.48	0.50	0.68	-	0.12	-	-	0.68
20	Imp-20	-	-	-	0.46	0.61	0.15	<D.L.	0.29	0.78	0.98	0.27	0.08	0.39	0.55	0.61	0.98	0.55	0.20	0.81	1.77	2.21
21	Imp-21	0.05	<D.L.	0.07	0.90	1.13	0.25	0.05	0.45	1.07	1.34	0.43	0.11	0.65	1.02	1.17	1.46	0.76	0.28	1.07	2.27	2.84
22	Imp-22	-	-	<D.L.	1.12	1.40	0.24	0.06	0.46	1.34	1.68	0.48	0.08	0.74	1.28	1.47	1.84	0.92	0.29	1.35	2.91	3.64
23	Imp-23	-	-	-	0.29	0.44	<D.L.	<D.L.	0.16	0.48	0.63	0.15	-	0.27	0.35	0.43	0.63	0.54	0.13	0.82	2.07	2.59
24	Imp-24	-	-	-	0.26	0.41	-	-	0.08	0.32	0.47	0.09	-	0.16	0.30	0.36	0.51	-	-	-	-	0.51
25	Imp-25	-	-	-	0.29	0.44	0.21	0.10	0.31	1.14	1.43	-	-	-	0.28	0.28	1.43	0.73	0.28	0.98	2.23	2.79
26	Imp-26	-	-	<D.L.	0.41	0.56	-	-	-	-	0.56	0.21	0.07	0.34	0.52	0.63	0.79	-	-	-	-	0.79
27	Imp-27	-	-	-	0.25	0.40	-	-	-	-	0.40	0.13	0.08	0.21	0.30	0.34	0.49	-	-	-	-	0.49
28	Imp-28	0.05	<D.L.	<D.L.	0.20	0.35	-	-	0.12	0.88	1.10	0.12	0.06	0.16	0.27	0.30	1.10	0.27	<D.L.	1.03	2.44	3.05
29	Imp-29	-	-	-	0.46	0.61	0.11	0.07	0.17	-	0.61	0.21	-	0.44	0.56	0.69	0.86	0.42	-	-	-	0.86
30	Imp-30	0.05	0.05	0.05	0.19	0.34	0.10	-	-	-	0.34	0.09	0.18	-	0.19	0.19	0.34	-	-	-	-	0.34

Table 3. *Cont.*

Peak	Peak Name	Initial Time, 25 °C					6 Months, 25 °C					24 Months, 25 °C						6 Months, 40 °C				
		AMP-Glucagon Lots			RLD *	EEC Upper Limit †	AMP-Glucagon Lots			RLD *	EEC Upper Limit †	AMP-Glucagon Lots			RLD*		EEC Upper Limit †	AMP-Glucagon Lots			RLD *	EEC Upper Limit †
		021914	021914A	021914B	C464 145A		021914	021914A	021914B	C215 547C		021914	021914A	021914B	C304 844A	C304 844C		021914	021914A	021914B	C215 547C	
31	Glucagon	99.2	99.4	99.3	91.7	83.7	97.1	98.8	95.8	88.1	83.7	95.6	98.2	93.9	89.6	88.4	83.7	92.1	97.3	89.1	70.3	83.7
32	Des-1	-	-	-	0.42	0.57	0.23	0.13	0.22	0.55	0.70	0.32	0.24	0.41	0.64	0.63	0.80	0.39	-	0.57	1.55	1.94
33	Des-2	0.07	<D.L.	-	0.40	0.55	0.18	0.05	0.16	0.56	0.71	0.13	0.14	0.32	0.62	0.63	0.79	0.33	0.06	0.54	1.56	1.95
34	Des-3	-	-	<D.L.	0.25	0.40	0.08	<D.L.	0.11	0.46	0.61	0.19	0.10	0.28	0.52	0.54	0.69	0.27	0.13	0.50	1.37	1.71
35	Des-4	-	-	-	0.18	0.33	0.05	<D.L.	0.07	0.43	0.58	0.13	0.05	0.17	0.40	0.43	0.58	0.18	0.10	0.34	1.13	1.41
36	Imp-31	-	-	-	-	0.15	-	-	-	0.08	0.23	-	-	<D.L.	0.09	0.09	0.24	-	-	0.06	0.16	0.31
37	Imp-32	-	-	-	-	0.15	-	-	-	-	0.15	-	-	-	-	-	0.15	-	-	0.06	0.37	0.52
NOI **, Observed		8	3	5	27	N/A	17	10	22	26	N/A	23	17	22	27	27	N/A	22	14	23	24	N/A
NOI **, ≥0.5%		0	0	0	3	N/A	0	0	0	9	N/A	0	0	3	9	10	N/A	6	0	11	15	N/A
All Imp. > 0.5%		0.0	0.0	0.0	3.7	4.6	0.0	0.0	0.9	8.7	10.9	0.5	0.0	1.9	7.1	7.8	10.9	4.1	0.0	9.2	25.3	31.6
Highest single		0.14	0.14	0.12	1.12	1.4	0.25	0.16	0.46	1.34	1.7	0.48	0.24	0.74	1.28	1.47	1.8	0.92	0.29	1.35	2.91	3.6
All Desamino		0.07	0.00	0.00	1.25	14	0.54	0.18	0.56	2	14	0.77	0.53	1.18	2.18	2.23	14	1.17	0.29	1.95	5.61	14
All Other Imp.		0.5	0.3	0.4	6.9	8.6	1.9	0.7	3.3	9.4	11.8	3.5	1.2	4.8	8.0	9.2	11.8	6.3	2.0	8.9	21.5	26.9
Total Impurities		0.6	0.3	0.4	8.2	31	2.5	0.9	3.9	11.4	31	4.2	1.7	6.0	10.2	11.4	31	7.4	2.3	10.9	27.1	31
Purity (Glucagon)		99.2	99.4	99.3	91.7	83.7	97.1	98.8	95.8	88.1	83.7	95.6	98.2	93.9	89.6	88.4	83.7	92.1	97.3	89.1	70.3	83.7

Imp: Unknown impurity. Des: Desmido impurity. D.L.: Detection Limit (0.05%). * RLD refers to ELI-Glucagon. ** NOI: Number of Impurities. † EEC lower limit is used for purity evaluation for Peak-31 (Glucagon). Any impurity percentage that is within the EEC upper limit is shaded green.

Overall, the synthetic glucagon for injection (AMP-glucagon) has an averaged purity (glucagon peak area) of 92.8–99.3% (Table 4). In comparison, the referenced drug (recombinant ELI-glucagon), has an averaged purity of 70.3–91.7% under the same storage conditions. It appears that at high temperatures or accelerated conditions (i.e., 40 °C), the stability of both AMP-glucagon and ELI-glucagon samples decreased. For example, the averaged purity of AMP-glucagon under 6 months at 25 °C was 97.2%, but the percentage decreased to 92.8% when stored at elevated stress conditions (40 °C). Nonetheless, the purity profile of AMP-glucagon at this accelerated temperature was still high, at 92.8%, when compared to the referenced ELI-glucagon, which was 70.3%.

Table 4. Summary of the purity and impurity profiles (%) for glucagon for injection under various stability conditions.

Type of Purity/ Impurity Analysis	Products	Time and Temperature, (as Mean ± S.D.) *			
		Initial Time	6 Months, 25 °C	24 Months, 25 °C **	6 Months, 40 °C
Purity	AMP-glucagon	99.3 ± 0.1	97.2 ± 1.5	95.9 ± 2.2	92.8 ± 4.1
	ELI-glucagon	91.7	88.1	89.0 ± 0.8	70.3
Total Impurities	AMP-glucagon	0.4 ± 0.1	2.4 ± 1.5	4.0 ± 2.2	6.9 ± 4.3
	ELI-glucagon	8.2	11.4	10.8 ± 0.83	27.1
Impurities with Area > 0.5%	AMP-glucagon	0.0 ± 0.0	0.3 ± 0.5	0.8 ± 1.0	4.4 ± 4.6
	ELI-glucagon	3.7	8.7	7.5 ± 0.6	25.3
Highest single Impurity	AMP-glucagon	0.13 ± 0.01	0.29 ± 0.15	0.49 ± 0.25	0.85 ± 0.53
	ELI-glucagon	1.12	1.34	1.38 ± 0.13	2.91
All Desamido Impurities	AMP-glucagon	0.02 ± 0.04	0.4 ± 0.2	0.8 ± 0.3	1.1 ± 0.8
	ELI-glucagon	1.3	2.0	2.2 ± 0.04	5.6
All Other Impurities	AMP-glucagon	0.4 ± 0.1	2.0 ± 1.29	3.2 ± 1.8	5.7 ± 3.5
	ELI-glucagon	6.9	9.4	8.6 ± 0.8	21.5

* For ELI-glucagon, only one lot was tested except for the 24 months. Therefore, data represent the value from the single lot. ** Samples were stored at room temperature ~20–25 °C for ELI-glucagon per labeling instruction.

For the equivalence evaluation of the impurity peaks, the EEC upper limit was calculated per Equation (2) and provided in Table 3, in which any impurity percentage within the EEC limit is shaded green. A total of 432 impurity evaluations for three AMP-glucagon lots at four different storage conditions were performed, and all met the EECs as indicated in Table 3. For "all desamido impurities" and "total impurities", an equivalent evaluation was based on the USP monograph, and all met the specifications of no more than 14 and 31%, respectively. In fact, the percentage of desamido impurities in AMP-glucagon were less than 2% under all stability conditions.

The equivalence evaluation of the purity profile of AMP-glucagon was also conducted. The main peak for glucagon (Peak-31) was used to evaluate the purity and its lower EEC was calculated based on Equation (1). All AMP-glucagon sample lots meet the purity EECs of 83.7%, as shown in Table 3.

One limitation of this study involves the 32 impurities that were unknown. Since peptide impurities can critically affect the quality, safety, and efficacy of drugs, a further study is needed to accurately identify, confirm, and quantify each impurity present in the glucagon products. Liquid chromatography coupled to high resolution mass spectrometry (LC/HRMS) has emerged as a key technique for the identification and quantification of structurally related peptide impurities [19], and therefore may be used to further characterize and identify the unknown impurities present in the synthetic and recombinant glucagon drug products.

4. Conclusions

In this study, a total of 42 parameters (4 desamido impurities, 32 unknown individual impurities, 5 grouping impurity profiles, and a purity profile) characterizing the impurity and purity profiles of AMP-glucagon and ELI-glucagon were analyzed under various stability conditions. Based on the study results, it can be concluded that the impurity profile for the synthetic glucagon for injection (AMP-glucagon) has a comparable and even lower level of impurities than the recombinant version (ELI-glucagon) under all stability conditions. AMP-glucagon has less impurities, with a peak area of >0.5% than ELI-glucagon in the entire storage life (24 months).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/scipharm90020032/s1, Figures S1–S4: Glucagon Impurity Profile by UPLC at Zero Time, 6 Months at 25 °C, 24 Months, and 6 Months at 40 °C, respectively; Table S1: Retention Times of UPLC for Glucagon Samples; Table S2: Calculated Relative Retention Times of UPLC for Glucagon Samples.

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