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Development of Analysis and Validation of Human Insulin-Containing Matrices Using Reverse Phase-Liquid Chromatography

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Abstract: Licensed drugs containing human insulin are used as a routine therapy to maintain blood glucose levels in patients with diabetes mellitus. Meanwhile, production and storage factors can reduce the potency of these drugs, and this is important for patients. Therefore, a fast and reliable analytical method is needed to ensure quality, as well as safety and effectiveness without being affected by the matrices content, such as m-cresol and glycerol. The study was developed with the high-performance liquid chromatography using a reverse-phase column Reliant™ C-18 (4.6 x 150 mm, 5 µm), 40°C column temperature, 215 nm UV-Vis detector, 1 mL/min flow rate, and 20 µL injection volume, while, ethylparaben was used as an internal standard. Furthermore, the separation was conducted with solution A and B containing Na_2SO_4 pH 2.3 and Na_2SO_4 pH 2.3 in acetonitrile (55:45, v/v) in a ratio of 38:62, v/v, while validation was performed according to ICH guideline. The human insulin retention time was 5.04±0.009 minutes, with linearity of 9.74-146.03 µg/mL (r = 0.9997). Accuracy and precision were 100.71±1.11% and 0.64%, while LOD and LOQ were 0.193 µg/mL and 0.643 µg/mL respectively. Human insulin remained stable at 23°C for 48 hours as demonstrated by ANOVA, $\alpha_{0.05.}$ Based on the results, this method has the potential to separate human insulin from A-21 desamido insulin as a degradant and matrices, as well as allowing for testing many products in a short time.

Keywords: Human insulin; Validation; Meta-cresol and glycerol; A-21 desamido insulin; Ethylparaben.

Introduction

The World Health Organization (WHO) and International Diabetes Federation (IDF) both define diabetes mellitus as a metabolic syndrome characterized by chronic hyperglycemia due to impaired insulin action (Atlas, 2019; Shen et al., 2019). Meanwhile, insulin is a hormone secreted by pancreatic β cells (Baeshen et al., 2014; De Haro Moreno et al., 2018; Shen et al., 2019) that regulates blood sugar levels and fat metabolism (Adams et al., 2018; Baeshen et al., 2014; De Haro Moreno et al., 2018). According to the literature, insulin is a peptide hormone made up of 51 amino acids (Adams et al., 2018; Baeshen et al., 2014; Norman & Litwack, 1997; Tokarz et al., 2018) sandwiched between two peptides (Najjar et al., 2014). It has a molecular weight of approximately 6000 Da (Judák et al., 2018; Norman & Litwack, 1997; Zuben et al., 2020), with the A and B chains having 21 and 30 amino acid residues, respectively (Adams et al., 2018; de Haro Moreno et al., 2018; Norman & Litwack, 1997; Tokarz et al., 2018). Human insulin preparations contain extra ingredients such as meta-cresol, glycerol, and zinc chloride in water for injection. The inclusion of two zinc ions in the formulation converts insulin dimer

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molecules into hexamers leading to product stability in the presence of m-cresol (Jacob et al., 2018) and glycerol. Furthermore, human insulin is degraded by deamidation (hydrolysis) reactions or polymerization when stored in non-compliant conditions (Najjar et al., 2014) leading to primary degradation of A-21 desamido insulin.

The British Pharmacopoeia, 2016 and The United States Pharmacopeial, 2019 both recommend reverse-phase high-performance liquid chromatography (RP-HPLC) for human insulin analysis, as do Moses A et al., 2019. However, according to several studies, this method is time-consuming (Iyire et al., 2018), as well as has an unsatisfactory peak, resolution and tailing (Najjar et al., 2014). Therefore, previous studies have contributed to the development and validation of human insulin assays that are both isocratic and gradient (Iyire et al., 2018; Moslemi et al., 2003; Moussa et al., 2010; Najjar et al., 2014; Ravi et al., 2007; Zuben et al., 2020). Although glycerol is a common excipient in pharmaceutical products containing human insulin, no study has described nor investigated its effect together with meta-cresol and A-21 desamido insulin. The presence of A-21 desamido insulin also needs to be investigated because it is the main degradant in human insulin.

Therefore, this study aims to develop and validate an analytical method using m-cresol, glycerol, and A-21 desamido insulin to obtain better retention time, resolution, and sensitivity than previous publications. The tests were carried out using RP-HPLC with commonly used detectors, while ethylparaben was used as an internal standard. This study assumed that the retention time of human insulin is moderate to maintain the principle of separation in HPLC.

Methods

Materials

The materials used include human insulin USPRS (Sigma Aldrich, cat.1342106) and ethylparaben (Tokyo Chemical Industry, cat.H0211). The water used was made with Milli-Q® apparatus and the water for injection (Otsuka). Other materials include HPLC grade acetonitrile (Merck, cat.100030), m-cresol for synthesis (Merck, cat.809691), anhydrous sodium sulphate (Merck, cat.106649), 37% hydrochloric acid (Merck, cat.100314), 85% ortho-phosphoric acid (Merck, cat.100573), methanol (Merck, cat.106009), 85% glycerol (Merck, cat.104094), PVDF membrane 0.45 µL 47 mm (Merck, Durapore® HVLP04700), and PTFE membrane 0.45 µL 25 mm (cat.51905268) at Agilent.

Apparatus

High-performance liquid chromatography instrument (Shimadzu/LC 20 AD SPD-20A Prominence/ L20104405073AE) was outfitted with LC–20AD LC pump, and SIL-20A HT autosampler (Shimadzu, serial number L20345002270). Moreover, fume hood (Esco Frontier Acela® EFA-4UDRVW-8), analytical balance (Mettler Toledo ML304T), micro-scale (Mettler Toledo XS 3DU), pH meter (Mettler Toledo seven multi-1230265386), degasser (Bransonic 3510 E-MTH), hotplate and stirrer (Thermo Scientific HPS-RT2 Advance), micropipettes (Eppendorf 2 µL, 200 µL, 1000 µL), and various glassware were used.

Solutions Preparations:

Mobile phase

Solution A was prepared from 56.8 g of anhydrous sodium sulphate in 2 L water of Milli-Q®, and a pH of 2.3 was adjusted by 85% ortho-phosphoric acid. Solution B was obtained by mixing solution A with acetonitrile in a ratio of 55:45, v/v. Both solutions were then filtered with a PVDF filter and sonicated for 15 minutes to remove gas before storing in dark bottles.

Ethylparaben

The stock solution was made by dissolving 2 mg of ethylparaben with methanol in a 10-mL volumetric flask.

Human Insulin

A total of 9.735 mg of human insulin standard was dissolved with 0.01 N HCl in a 20-mL volumetric flask to obtain a concentration of 486.75 µg/mL of stock solution. The stock was filtered through a PTFE membrane and then kept at a temperature ranging from 2-8°C for not more than 48 hours.

A-21 desamido insulin

A total of 2.5 mL human insulin stock solution was placed at 23°C for 3 days to obtain at least 5% A-21 desamido insulin. Each day, the solution was injected into the HPLC system to check the percentage.

Meta-cresol

Stock Solution I namely 97 µL of m-cresol (1.03 kg/L) was dissolved with 0.01 N HCl in a 20-mL volumetric flask. Stock Solution II consisting of 0.1 mL of stock I was pipetted and added with 0.01 N HCl in a 10-mL volumetric flask.

Matrices

The matrices stock solution was prepared by mixing 1 mL of m-cresol stock II and 0.3 mL of 85% glycerol (1.23 kg/L) and then adding water for injection in a 25-mL volumetric flask. One mL of the matrices solution was used to generate solutions for linearity,

specificity, accuracy, precision, quantitation limit (LOQ), detection limit (LOD), and stability assays.

Resolution

The resolution solution was produced to validate the method on specificity assay. A total of 0.5 mL ethylparaben stock, 2.057 mL A-21 desamido insulin stock, and 2 mL of m-cresol II stock were mixed in a 5-mL volumetric flask, added with 0.01 N HCl, and then filtered through PTFE membrane. The resolution solution consists of approximately 200 µg/mL human insulin containing more than 5% A-21 desamido insulin, 20 µg/mL ethylparaben, and 20 µg/mL m-cresol.

Method development

The isocratic analytical conditions were developed using a reverse-phase chromatography with Reliant™ C-18 column (4.6 x 150 mm, 5 µm) set at 40°C to separate the analytes. Furthermore, UV-VIS detector at 215 nm, 20 µL injection volume, and 1 mL/min flow rate were conducted throughout the study. The mobile phases A and B were operated with a ratio of 38:62, v/v, while the retention time, tailing factor, resolution, and peak area were also recorded.

System suitability test (SST)

The SST was performed by injecting a solution consisting of three analytes namely 115 µg/mL human insulin, at least 5% A-21 desamido insulin, and 10 µg/mL ethylparaben five times according to the analytical conditions. The percentage of A-21 desamido insulin was calculated from its area divided by the sum of areas of human insulin and A-21 desamido insulin. Meanwhile, the area ratio (AR) was obtained from the total area of human insulin and A-21 desamido insulin divided by the area of ethylparaben, then the mean and SD of AR were calculated to determine the percentage of relative standard deviation (%RSD).

Validation:

Validation was carried out according to the parameters of linearity, specificity, accuracy, precision, LOD and LOQ, as well as stability (European Medicines Agency, 2011; FDA, 2003; Harron, 2013) to ensure the dependability of analytical procedures in obtaining valid data. All test solutions used were produced using 0.01 N HCl in a 5-mL volumetric flask and filtered through a PTFE membrane. Calculation of the analysis was carried out using the area ratio (AR), obtained by dividing the area of human insulin by the area of ethylparaben.

Specificity

The specificity test was conducted using a blank, 20 µg/mL ethylparaben, matrices, 20 µg/mL m-cresol, and resolution solutions. The resolution solution was injected in triplicate, while the others were in duplicate.

Standard Curve

The standard curve was obtained from seven solutions spiked with human insulin in the range of 9.74 - 146.03 µg/mL, each containing 10 µg/mL ethylparaben. Each test solution was injected in triplicate, then a regression line was plotted for human insulin concentration as the X-axis versus the area ratio as the Y-axis. This curve was used in calculations for accuracy, precision, stability, and product determination assays.

Linearity

The linearity test was conducted using seven solutions spiked with human insulin in the range of 9.74 - 146.03 µg/mL, each containing 10 µg/mL ethylparaben, and 1 mL matrices solution. Each concentration was produced in two series in different volumetric flasks. The regression line was created similarly as standard curves.

Accuracy and precision

Accuracy and precision tests were carried out under the same conditions of personnel, time, equipment, and condition. The Association of Offical Analytical Chemists (AOAC) 2016 criteria were used as a guideline for determining the acceptability. The investigation used solutions spiked with 24.338 µg/mL, 73.013 µg/mL, and 121.688 µg/mL human insulin, containing 10 µg/mL ethylparaben and 1 mL matrices solution, respectively. Each concentration was made in three series in different volumetric flasks. The percentage of recovery (%R) and %RSD was calculated using the standard curve.

LOD and LOQ

The LOD and LOQ test solutions were prepared in five solutions spiked with 0.487 - 5.841 µg/mL of insulin human, which contained 10 µg/mL ethylparaben and 1 mL matrices solution, respectively. The solution was injected in duplicate. Detection and quantitation limits were calculated, and a calibration curve was formed.

Stability

Stability tests were carried out during the analysis period and storage according to FDA guidance document Q1A(R2) , 2003. Three solutions spiked with human insulin 24.338 µg/mL, 97.35 µg/mL, and 146.03 µg/mL, containing 10 µg/mL ethylparaben and 1 mL matrices solution, respectively were used. Each

concentration was produced in three series in different volumetric flasks. They were stored at 23°C and injected at 0 (fresh), 12, 24, and 48 hours. Furthermore, the %R was calculated using a standard curve, while the stability was tested statistically by ANOVA $\alpha_{0.05}$.

Determination of human insulin products levels on the market

Three different packages of the same Actrapid® batch produced by Novo Nordisk were quantitatively tested. A total of 2 mL of sample was mixed with 5.0 µL of 9.6 N HCl and shaken slowly. Subsequently, 0.1425 mL was placed in a 5-mL volumetric flask containing 10 µg/mL ethylparaben, then added with 0.01 N HCl. The test was carried out in duplicate. A standard curve was used to calculate potency claims.

Result and Discussion

System suitability test (SST)

The mean of retention times of human insulin, A-21 desamido insulin, and ethylparaben were 5.03±0.009 minutes, 6.10±0.008 minutes, and 12.69±0.003 minutes, respectively (Table 1). The %RSD of the AR value was 0.44%, which is less than 2% (Naik et al., 2018; Swamy et al., 2014; Swathi et al., 2017). The SST focuses on determining the performance (Ganji & Satyavati, 2015) and reproducibility of the chromatography system based on the analysis carried out according to the results of the development (Zuben et al., 2020). The %RSD of the AR value of 0.44% indicating that the level of accuracy in the SST test is acceptable.

This shows that the solvent, column conditions, instrument, and mobile phase used are suitable for analytical purposes. According to Barth HG, 2019, the resolution solution was made to determine whether the analytes involved can be well separated and have a resolution not less than 1.5 using the developed analytical conditions.

Table 1. Human Insulin System Suitability Test Results

Analyte	Area	Retention Time	Tailing Factor	A-21 desamido (%) *	Area Ratio (AR) †
Human insulin	1,702,344	5.025	1.065	5.660	2.650
	1,705,159	5.040	1.063	5.640	2.660
	1,706,804	5.026	1.067	5.640	2.670
	1,689,753	5.016	1.067	5.690	2.650
	1,686,723	5.034	1.067	5.680	2.630
A-21 desamido insulin	102,079	6.090	1.095		
	101,838	6.107	1.095		
	102,082	6.101	1.093		
	101,854	6.093	1.093		
	101,507	6.108	1.096		
Ethylparaben	680,543	12.684	1.057		
	680,201	12.684	1.058		
	678,743	12.690	1.057		
	677,325	12.685	1.057		
	678,914	12.689	1.057		
Mean				5.660	2.650
SD				0.020	0.010

*: A-21 desamido insulin area divided by the sum of human insulin and A-21 desamido insulin area

†: Sum of human insulin and A-21 desamido insulin divided by the ethylparaben area

Specificity

Based on the specificity test results, the retention times of human insulin, A-21 desamido insulin, m-cresol, and ethylparaben were 5.04±0.009 minutes, 6.10±0.018 minutes, 10.26±0.001 minutes, and 12.69±0.002 minutes, while their resolutions were 9.24±0.043, 1.78±0.005, 8.09±0.056, and 5.43±0.007, respectively (figure 1). The data were expressed as mean and standard deviation (n=3). In this study, the matrices had retention time equivalent to m-cresol, because the glycerol peak did not appear.

The figure demonstrates that the blank, human insulin, A-21 desamido insulin, m-cresol, and ethylparaben were completely separated, with sharp and perpendicular peaks. The figure illustrates that this analytical technique can separate human insulin from its main degradation namely A-21 desamido insulin, and m-cresol, using an internal standard of ethylparaben without interfering with the interactions between the various components. In addition, glycerol presence did not interfere with the analysis of human insulin and other analytes.

According to Najjar A et al., 2014, human insulin retention time was 7.9 minutes with resolution of A-21 desamido was 1.56. Therefore, the obtained human insulin retention time of 5.04 minutes with a resolution of A-21 desamido of 1.78 indicates that the method developed in this study separates the peaks better at a fast time and the peaks were completely separated.

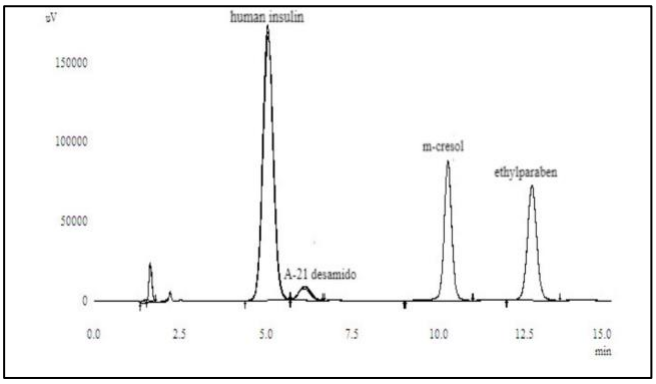

Figure 1. Chromatogram of resolution solution in human insulin specificity test

Standard curve and linearity

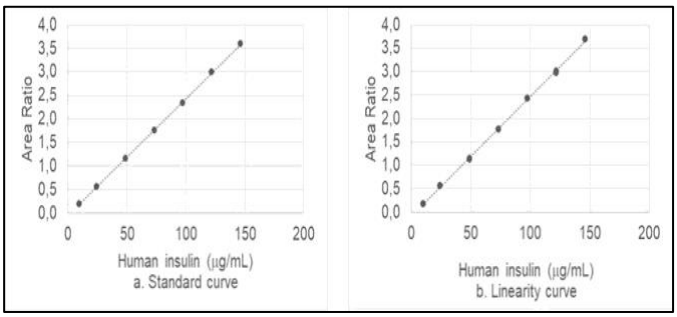

Figure 2. Calibration curves of human insulin (9.74 –146.03 µg/mL)

Seven different solutions containing 9.74 - 146.03 µg/mL human insulin without matrices produced a standard curve with the equation of the line y = 0.025x - 0.0608, while the correlation coefficient value was 0.9999 (figure 2a). Meanwhile, the other seven solutions containing the matrices produced linearity curves as shown in Figure 2b. The equation of the line is y = 0.0256x - 0.087 with a correlation coefficient of 0.9997. The presence of glycerol and m-cresol in the investigated human insulin concentration also produced linear results, the linearity and standard curves were apparently similar.

Accuracy and precision

The real human insulin concentration (rC) was calculated using the standard curve (Table 2a). The %R range calculated with the gains for the respective concentrations was 100.97-102.46%, 100.12-100.52%, and 99.16-100.86%. Meanwhile, the accepted requirements of %R from AOAC, 2016 for 24.338 µg/mL and 73.013 µg/mL ranges from 90-107%, while that of 121.688 µg/mL is between 95-105%. Based on the results, the average %R is 100.71±1.11%. Furthermore, the average %RSD of rC obtained for precision was 0.64%, while the %RSD acceptability for 24.338 µg/mL and 73.013 µg/mL were <7.3%, and for 121.688 µg/mL was <5.3%.

The average %R in accuracy assay, indicating that the method used to analyse human insulin was highly accurate, producing a level measurement that was significantly close to the actual value. The precision results show that the %RSD listed in Table 2a on the investigated concentration produced a good and satisfactory consistency. The accuracy and precision tests are acceptable when compared to the AOAC, 2016.

LOD and LOQ

According to Table 2b, the LOD and LOQ values were 0.193 µg/mL and 0.643 µg/mL, respectively. Furthermore, the intercept value obtained was -0.0083 and the slope was 0.0194 with a calculated correlation coefficient of 0.998. The test solution was prepared with the lowest possible human insulin level to determine the true limits of detection and quantitation that can be achieved by the instrument analytical conditions. Najjar A et al., 2014 obtained LOD and LOQ 2.93 µg/mL and 9.78 µg/mL respectively, while Iyire A et al., 2018 yielded 0.63 µg/mL and 2.0 µg/mL respectively. The results of this study show more sensitive yields in the investigated concentration. The presence of glycerol and m-cresol in low concentrations of human insulin produced good, valid, and reliable results. In addition, the obtained correlation coefficient on the standard curve, linearity curve, LOD and LOQ curve shows linear results.

Table 2. Accuracy, Precision, LOD And LOQ Of Human Insulin Test Results

(a) Accuracy and precision

C	Area		Area Ratio	rC	%R	%RSD
	Human insulin	Ethylparaben				
	443,435	791,549	0.560	24.886	102.25	
24.338	439,207	795,029	0.552	24.574	100.97	0.791
	444,547	791,745	0.561	24.936	102.46	

	1,395,458	788,057	1.771	73.395	100.52	
73.013	1,381,284	780,195	1.770	73.382	100.51	0.227
	1,379,695	782,397	1.763	73.101	100.12	
	2,319,387	783,435	2.961	121.072	99.49	
121.688	2,306,227	768,268	3.002	122.728	100.86	0.898
	2,315,577	784,820	2.950	120.669	99.16	

(b) LOD and LOQ

C	Area		Area Ratio
	Human insulin	Ethylparaben	
0.487	3,595	834,914	0.0043
	3,635	835,385	0.0044
0.974	6,485	829,195	0.0078
	6,323	828,304	0.0076
1.947	23,344	814,484	0.0287
	22,545	813,794	0.0277
3.407	48,660	812,994	0.0599
	47,067	813,827	0.0578
5.841	81,407	768,522	0.1059
	80,313	769,111	0.1044

rC: Real human insulin concentration (µg/mL)

C: Human insulin concentration (µg/mL)

Stability

The stability test results of are shown in Table 3. The P-value obtained was 0.56 demonstrated by ANOVA $\alpha_{0.05}$. The mean of %R at 146.03 µg/mL was higher than other concentrations. Three spiked human insulin test solutions in stability assay were used to investigate potential variations and the p-value indicates that there was no significant difference in %R for 48 hours. Based on the results, human insulin was stable at 23°C during the analysis.

Table 3. Human Insulin Stability Test Result At 23°C For 48 Hours

C	% R ‡				Mean §	SD §
	Fresh	12 hours	24 hours	48 hours		
24.338	101.895 ± 0.806	102.803 ± 0.153	101.182 ± 0.688	99.837 ± 0.077	101.430	1.220
97.350	102.254 ± 0.393	103.505 ± 1.086	100.784 ± 0.072	100.177 ± 0.092	101.680	1.440
146.030	102.829 ± 0.134	102.796 ± 0.225	101.250 ± 0.104	100.994 ± 0.132	101.970	0.900

‡: Data are expressed as mean ± standard deviation (n=3)

§: Calculated from all %R for 48 h

Determination of human insulin products levels on the market

Table 4 shows the potency results of three product packages containing human insulin that are sold in the market. Products must have a potency percentage of 95-105%. In addition, the potency obtained in this study indicates that the sample meets the criteria for the range specified on the label. Cold chain and storage in the health facility where products are purchased were still optimal in maintaining product quality. Finally, this analytical method is suitable and useful for determining the potency of products containing human insulin.

Table 4. Pharmaceutical Dosage Levels Determination

Products	Area Ratio ‡	C ‡	% Potency ‡
1	2.45 ± 0.01	98.22 ± 0.30	99.32 ± 0.30
2	2.41 ± 0.00	96.60 ± 0.02	97.68 ± 0.02
3	2.40 ± 0.00	96.30 ± 0.10	97.38 ± 0.10

‡: Data are expressed as mean ± standard deviation (n=2)

Analysis of human insulin by RP-HPLC often does not match the factors of the column, mobile phase, and temperature. The results obtained had blunt and branching peaks, unstable retention times, as well as poor resolution of A-21 desamido insulin. This result is considered the most appropriate under conditions of analysis developed in the presence of human insulin, matrices, A-21 desamido insulin, and ethylparaben. The use of dark bottles as mobile phase container play an important role in maintaining the retention time of the analytes, especially human insulin as they prevent light from interfering with the analysis. Meanwhile, the colorless bottles used in this study as the mobile phase containers have potential risks in human insulin analysis, due to the drastically variable retention times. Furthermore, ethylparaben was used as the internal

standard because it is stable, compatible with column and other analytes, has a relatively short retention time, separates effectively, and is readily available.

Conclusion

Optimal chromatographic separation was obtained using isocratic analytical conditions developed by investigating the presence of glycerol together with m-cresol and A-21 desamido insulin. Human insulin has a very fast retention time of 5.04 minutes and can be separated from its degradant with a good resolution of 1.78. Furthermore, the use of ethylparaben as an internal standard helped to produce a satisfactory validation test. Based on the results, this analytical method can be used in other laboratories that require fast and accurate analysis.

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