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 Na₂SO₄ pH 2.3 and Na₂SO₄ 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 (r² = 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, α=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...

How to Cite:
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.
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**

Table 1. Human Insulin System Suitability Test Results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Area</th>
<th>Retention Time</th>
<th>Tailing Factor</th>
<th>A-21 desamido (%) *</th>
<th>Area Ratio (AR) †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human insulin</td>
<td>1,702,344</td>
<td>5.025</td>
<td>1.065</td>
<td>5.660</td>
<td>2.650</td>
</tr>
<tr>
<td></td>
<td>1,705,159</td>
<td>5.040</td>
<td>1.065</td>
<td>5.640</td>
<td>2.660</td>
</tr>
<tr>
<td></td>
<td>1,706,804</td>
<td>5.026</td>
<td>1.067</td>
<td>5.640</td>
<td>2.670</td>
</tr>
<tr>
<td></td>
<td>1,689,753</td>
<td>5.016</td>
<td>1.067</td>
<td>5.690</td>
<td>2.650</td>
</tr>
<tr>
<td></td>
<td>1,686,723</td>
<td>5.034</td>
<td>1.067</td>
<td>5.680</td>
<td>2.630</td>
</tr>
<tr>
<td>A-21 desamido insulin</td>
<td>102,079</td>
<td>6.090</td>
<td>1.095</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>101,838</td>
<td>6.107</td>
<td>1.095</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>102,082</td>
<td>6.101</td>
<td>1.093</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>101,854</td>
<td>6.093</td>
<td>1.093</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>101,507</td>
<td>6.108</td>
<td>1.096</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylparaben</td>
<td>680,543</td>
<td>12.684</td>
<td>1.057</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>680,201</td>
<td>12.684</td>
<td>1.058</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>678,743</td>
<td>12.690</td>
<td>1.057</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>677,525</td>
<td>12.685</td>
<td>1.057</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>678,914</td>
<td>12.689</td>
<td>1.057</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean SD 5.660 0.020 2.650 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.

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

<table>
<thead>
<tr>
<th>C</th>
<th>Human insulin</th>
<th>Ethylparaben</th>
<th>Area</th>
<th>Area Ratio</th>
<th>%R</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.338</td>
<td>443,435</td>
<td>791,549</td>
<td>0.560</td>
<td>24.886</td>
<td>102.25</td>
<td>0.791</td>
</tr>
<tr>
<td></td>
<td>439,207</td>
<td>795,029</td>
<td>0.552</td>
<td>24.574</td>
<td>100.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>444,547</td>
<td>791,745</td>
<td>0.561</td>
<td>24.936</td>
<td>102.46</td>
<td></td>
</tr>
</tbody>
</table>
Stability

The stability test results of are shown in Table 3. The P-value obtained was 0.56 demonstrated by ANOVA α = 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

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

‡: 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

<table>
<thead>
<tr>
<th>Products</th>
<th>Area Ratio ‡</th>
<th>C ‡</th>
<th>% Potency ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.45 ± 0.01</td>
<td>98.22 ± 0.30</td>
<td>99.32 ± 0.30</td>
</tr>
<tr>
<td>2</td>
<td>2.41 ± 0.00</td>
<td>96.60 ± 0.02</td>
<td>97.68 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>2.40 ± 0.00</td>
<td>96.30 ± 0.10</td>
<td>97.38 ± 0.10</td>
</tr>
</tbody>
</table>

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

**Acknowledgements**

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