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Validation of a new analytical method for the RP-HPLC quantitative analysis of recombinant human insulin

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Abstract

A rapid, accurate, dependable, and repeatable RP-HPLC method was created for the measurement of insulin. A sodium sulphate anhydrous buffer with a pH of 2.3 and acetonitrile (76:26) are used as the mobile phases A and B, respectively, in the most recent validated isocratic RP-HPLC analytical technique for the determination recombinant human insulin (RHI). The samples were separated using a 150 x 4.6 mm waters zorbax C18, flow rate of 1.0 ml/min, 3.5 m column. It was found that 214 nm is the wavelength at which ultraviolet detection occurs. In order to analyse the desamido breakdown product of insulin; this method was tested with phenol and m-cresol, which are preservatives used in commercial insulin formulations at low concentrations, and with clear separation between each peak. The technique was shown to be linear over the 40-60 g/ml concentration range with a regression coefficient of r^2 = 0.9994. During accuracy tests, it was revealed that the mean recovery was around 100.35 per cent. A low-cost, dependable, exact, linear, and quick RP-HPLC technique was created and verified in accordance with ICH criteria. As it has been demonstrated to be reliable, this method increasingly frequently used to evaluate human insulin.

1. Introduction

Diabetes mellitus and hyperglycemia brought on by a variety of reasons are both conditions that are treated with the medicine insulin. It is a member of the group of medications referred to as short-acting insulin. This exercise includes the daily insulin's a method of action, marked and unlabeled indications, side effect profile, contraindi-cations, the toxicity, tracking, and which are crucial for healthcare team members managing patients with diabetes and related illnesses (Baynest *et al.*, 2015). The two peptide chains, A and B, that make up human insulin have a respective amino acid count of 21 and 30, and they are joined by three disulfide connections. Assessment of C-peptide, and insulin immunoassays being indirect indicators with insulin abuse during a sport. Measuring equine insulin (Abellan *et al*., 2009; Bilal Yilmaz, *et al*., 2012) using a chemiluminescent immunoassay and measuring human insulin levels in insulin-dependent diabetes mellitus patients using high-performance liquid chromatography. As far as we are aware (Carslake *et al*., 2017; Chen *et al*., 2013), there is no published technique for quantitatively detecting insulin recombinant using RP-HPLC. The purpose of this work is to assess the (preservatives in commercial insulin). Desamido, a breakdown product of insulin-R using the RP-HPLC method (Arby *et al*., 2001; Malle *et al*., 2015).

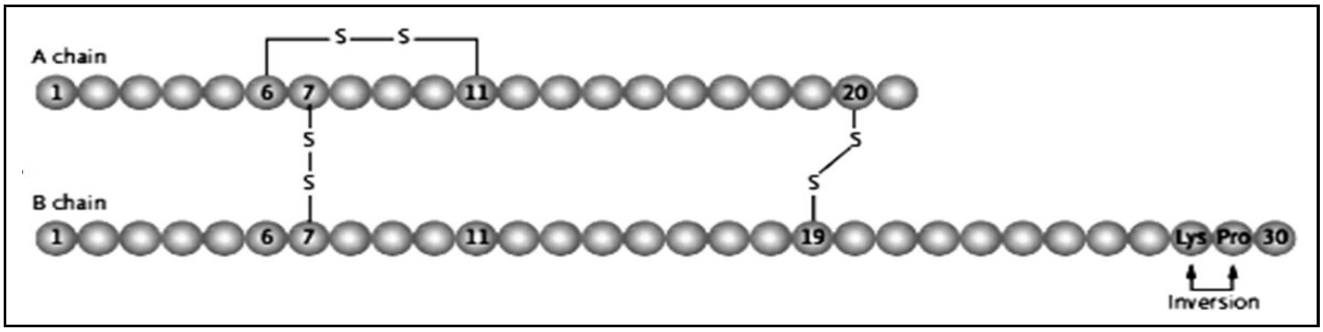

Figure 1 : Human insulin structure.

A disulfide bridge that joins the C and N terminal helices to A and B with a central terminal forms the link between the A and B chains. When triggered, the beta cells release insulin into the capillaries of the islet cells, where it is stored along with proinsulin, insulin, and the C-peptide (Taylor *et al*., 2016; McPhaul *et al*., 2016). It is the portal vein, which conducts the gastrointestinal tract's blood stomach towards the liver, receives the blood that these capillaries empty into. The amount of insulin in the blood will rise after being exposed to hyperglycemia for 30 min (Wahl *et al*., 2019). Fatty acids, amino

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acids, a number of hormones, and keto acids (produced as a result of fatty acid breakdown) secreted from the digestive system can also increase the production of insulin. Somatostatin inhibits the release of insulin as does sympathetic nervous system activation, which includes the fight-or-flight response. Insulin's main effect is to promote glucose uptake in the liver, muscles, and adipose (fat) tissues, all of which are important for nutrition metabolism and storage (Duraisami *et al*., 2021; Venkatachalam *et al*., 2021) As a result, the need for insulin will increase to around 16,000 kg per year, and the productivity of the current insulin expression systems would not be enough to meet these demands. New insulin delivery strategies, such as ingestion or inhalation, as well as more effective insulin expression techniques must be created.

The many strategies for the manufacture of insulin are covered in the current article (Amisha Sharma *et al*., 2021; Tim Heise *et al*., 2009; Bretzel *et al*., 2004; Jacobsen *et al*., 2000; Hermansen *et al*., 2004; Punit *et al*., 2019).

Diabetes patients did not live lengthy lives prior to the 1921 discovery of insulin. The most effective treatment involved putting diabetic patients on strict, diets that limit carbohydrates. Patients would not be fully cured, but they might survive a few more years as a result. Patients who were on limited diets of only 450 calories per day had passed away from hunger.

It is also possible to manufacture the A and B chains of insulin independently using a different two-chain technique. Proinsulin is sufficiently produced by *E. coli* that has undergone genetic modification. To create this recombinant protein, proinsulin-producing plasmids are introduced into *E. coli*. After that, kanamycin monosulphate antibiotic-containing tryptic soy broth is used to culture the altered cells. Together with the proinsulin coding genes, the plasmid also contains a kanamycin monosulphate resistance gene, allowing the transformed *E. coli* to thrive in the broth.

2. Materials and Methods

Alcohol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), sodium sulphate anhydrous (analytical grade), orthophosphoric acid solution (analytical grade), and ethanolamine (analytical grade) are all acceptable materials for analytical use.

2.1 Buffer preparation (solution A)

Anhydrous sodium sulphate was precisely measured at 56.8 g and transferred to a volumetric flask of 2000 ml. Then, after adding water to dissolve it, add water to equal the volume. A total of phosphoric acid was pipetted into the mixture in 5.4 ml, and if necessary, the pH was raised to 2.3 using ethanolamine.

2.2 The following actions are required to prepare the standard solution

The correct weight of approximately human insulin standard, 10 mg, was transferred to a 10 ml standard flask and dissolved in 0.01 M hydrochloric acid, diluted to the proper concentration with 0.01 M hydrochloric acid, and carefully mixed (1000 µg/ml).

2.3 Preparation of the sample solution (for the human insulin recombinant assay)

The contents of about 10 pool vials of material were transferred, and they were well mixed in a clean glass beaker. Using a pipette, 10.0 ml (10 mg) of the a 10 ml volumetric flask containing 40 ml of 0.01 M hydrochloric acid was filled with the combined sample solution was added to test whether it would be possible to see a suspension. If, a suspension did not become clear after the initial injection of acid, which took place after 5 min, small aliquots of a 0.01 M hydrochloric acid solution were added until a conclusive solution was reached.

Table 1: Chromatographic conditions

Mode of separation	Isocratic
Stationary phase	Zorbax 300SB-C18, 4.6 x 250 mm, 5 µm or equivalent
Detector wavelength	214 nm
Mobile phase	Solution A: Acetonitrile (74:26 %v/v)
Flow rate	1ml/min
Temperature	40°C
Sample load 10 µl	10 µl

2.4 Specificity

Specificity is the capacity to assess the analyte unambiguously in the presence of components that might be present. Common examples include impurities, degradants, matrix, and other compounds. We made blank, reference, and sample solutions (50 µg/ml).

2.5 Linearity

The linearity solutions were made by taking 0.40 ml, 0.45 ml, 0.50 ml, 0.55 ml, and 0.60 ml from the normal stock solutions of the sample, transferring these solutions into six separate 10 ml volumetric flasks, and diluting to 10 ml with diluents to obtain 40 µg/ml, 45 µg/ml, 50 µg/ml, 55 µg/ml. After each solution was injected, the chromatograms were captured at 214 nm. The concentration spectrum displayed above is linear and according to beer's rule. Plotting the peak areas versus concentration allowed for the calibration to be constructed.

2.6 Accuracy

The method's accuracy was examined by adding the drug standard at concentrations of 75, 100, and 125 per cent to a predefined tablet solution and determining percent recovery data.

2.7 Robustness

The capacity of a system to resist variations in chromatographic parameters, such as temperature 1°C, flow rate (0.1 ml), and the ratio of organic content to mobile phase (10:1) or other slight or deliberate modifications, is referred to as robustness. Chromatograms were recorded after injecting 10 of solution into the chromatographic apparatus for each condition. It was investigated how the device suitability criteria affected things.

3. Results

3.1 System suitability analysis

Retention time and peak area values from the device suitability analysis were found to be within acceptable bounds. Table 2 displays the results. In the specificity study, no interference from diluents all known impurities during the retention time of the insulin peak was seen.

3.2 Specificity

All known impurities were present at the retention time of the insulin peak in the specificity study without diluent interference. The chromatogram was shown in Figure 2.

3.3 Linearity

In order to assess linearity and produce a calibration curve, the graph between the acquired peak areas and concentrations was generated. With a slope of 71054 an intercept of 778154 for the correlation coefficient of r^2= 0.9989 was the regression equation obtained during the linearity determination. Calibration curve was shown in Figure 3. The report analysis was shown in Table 3.

3.4 Method precision

Human insulin estimate variance within and across days revealed that the % RSD was less than 2%. These low RSD values demonstrate the method's high degree of precision in Table 5.

3.5 Accuracy

The accuracy of the established approach was evaluated in terms of percent recovery studies at three distinct levels was 80% to 120% when a known volume of the standard medication was added to pre-analyzed samples and submitted to the suggested HPLC process .

3.6 Robustness

The results of the robustness analysis showed that small adjustments in flow rate, wavelength, and temperature had no impact on the factors selected. A regular and representative retention period was found to exist. The robustness values are shown in Table 5.

Table 2: System suitability test

Parameters	Human insulin	Desamido
Retention time	17.750	22.242
Peak	37890894	6597

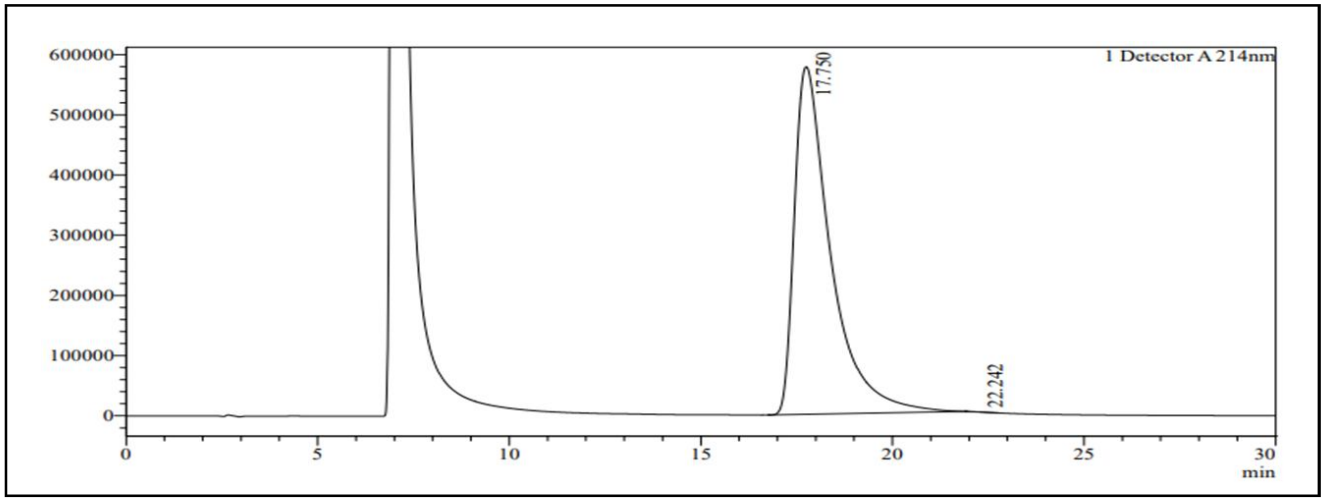

Figure 2: Chromatogram for specificity.

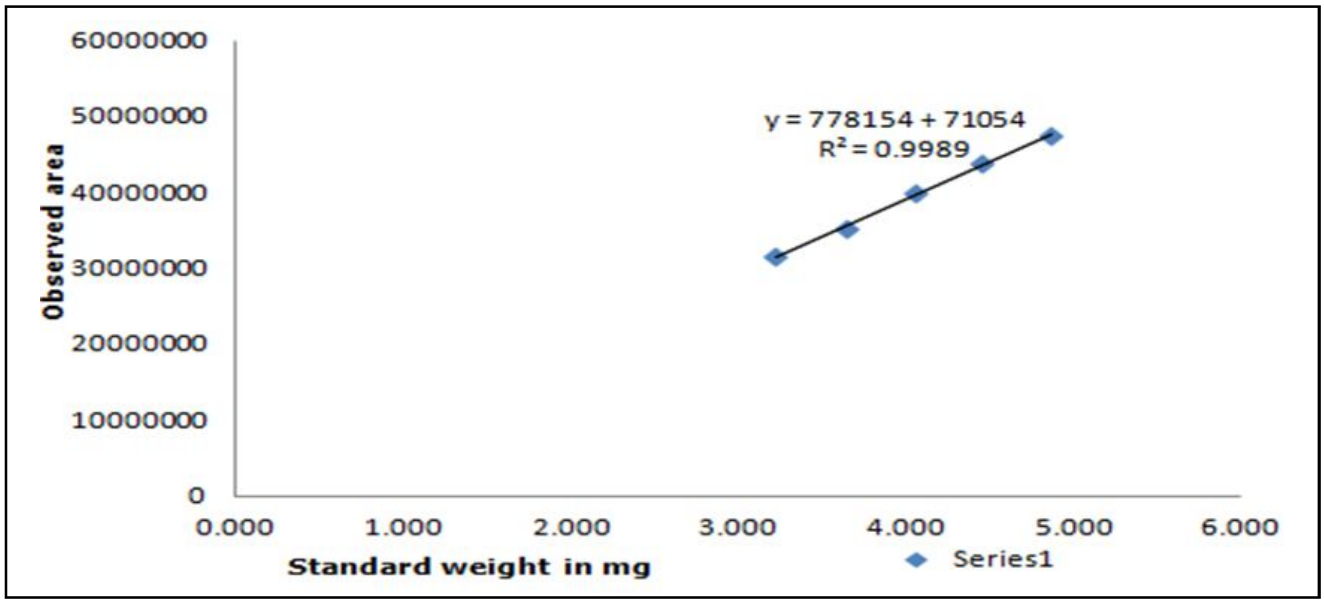

Figure 3: Linearity chromatogram.

Table 3: Linearity data for human insulin recombinant

Denomination in (mg/ml)	Observed area
3.224	31635645
3.642	35354354
4.053	39881817
4.453	43813350
4.863	47553431
Correlation coefficient	0.99950

Table 4: Accuracy data for human insulin recombinant

Concentration %	% recovered	Mean	%RSD
80	97.00		
90	98.20		
100	100.70	99.00	1.21
110	98.3		
120	097.20		

Table 5: Method precision data for human insulin recombinant

Method precision		
S.No.	Sample rhi	Content (% of label claim)
1	98.5730	98.6
2	98.6110	98.6
3	98.5550	98.6
4	98.8220	98.8
5	98.8810	98.9
6	98.8070	98.8
Mean		98.7
RSD (NMT 2.0%)		0.14

Table 6: Robustness data for human insulin recombinant

Parameters	Conditions	Retention time
Low flow rate (ml/min)	0.9 ml/min	19.333
High flow rate (ml/min)	1.1 ml/min	16.825
Low pHHigh pH	2.3 – 0.5 ml/min	18.242
	2.3 + 0.5 ml/min	18.392
Low column temperature	39°C	16.967
High column temperature	41°C	19.200

4. Discussion

System compatibility assessments, in accordance with the United States Pharmacopoeia, are an essential component of liquid chromatographic operations. For standard solutions, we quantified the theoretical plates' total number, area, and retention time. Chromatograms were obtained following the injection into the apparatus of six identical doses of a 50 g/ml human insulin solution.

Specificity is the capacity to reliably assess the analyte in the presence of possible components. Typical examples include impurities, degradants, matrix, and other compounds. The following remedies were created: blank, typical, and example. The chromatograms were recorded after solutions were added to the device.

The normal stock solutions of the sample were divided into six separate 10 ml volumetric flasks. Each of these flasks received 3 ml, 4 ml, 5 ml, and 6 ml of the linearity solutions, which were subsequently diluted to 10 ml using diluents to create 30 µl/ml, 40 µl/ml, 50 µl/ml, and 60 µl/ ml, respectively. Chromatograms were obtained at 214 nm after each solution injection. The concentration spectrum seen above is linear and conforms to beer's law. The calibration was created using a concentration vs peak area graph. The correlation coefficient (R) was found to be 0.9995, which is within the permitted ranges.

The accuracy of the approach was assessed by adding the drug standard to a prescribed tablet solution at concentrations of 80, 100, and 120% and determining percent recovery studies. Since the mean recovery values were 99.00%, very little interference from matrix elements is present.

Precision was estimated using inter-day precision studies. On the same day, six analyses of the same sample were carried out at a concentration of 4 µl/ml to check for any differences in the outcomes. Three days in a row were devoted to studying, with inter-day precision. The inter-day RSD values were found to be 0.14%, and the outcome was within the acceptable range.

The ability of a system to survive subtle or deliberate changes in chromatographic conditions, such as pH, flow rate (0.1 ml/min), and temperature (1°C), is known as robustness. 10 µl/ml of the substance's solution, which is within limits, were added to the chromatographic for each circumstance.

5. Conclusion

A quick analytical, reliable, and isocratic RP-HPLC method was created to measure Insulin recombinant. The established analytical method's validation produced outcomes that were within the parameters of the ICH guidelines. A validated RP-HPLC method for separating insulin and its desamido breakdown product was used to separate insulin recombinant.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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