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NOVEL ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE QUANTITATIVE ANALYSIS OF INSULIN R BY RP-HPLC

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ABSTRACT

For the estimation of Insulin, a quick, precise, reliable, and repeatable RP-HPLC method was developed. The latest established isocratic RP-HPLC analytical method for determining insulin uses a pH 2.3 sodium sulphate anhydrous buffer as mobile phase-A and acetonitrile (76:26) as mobile phase-B. Waters, Zorbax C18, (150X4.6mm), 3.5m column with a flow rate of 1.0 mL/min was used to separate the samples. The wavelength of ultraviolet detection was discovered to be 214 nm. This method was tested in the presence of phenol and m-cresol, which are present in low concentration in commercial insulin preparations as preservatives, with good distinction between their peaks, for the study of insulin and its desamido degradation product, as well as in the presence of phenol and m-cresol, which are present in low concentration in commercial insulin preparations as preservatives. With coefficient regression r2 = 0.9994, the procedure was found to be linear over the concentration range 40-60 g / ml. During accuracy tests, the mean recovery was found to be in the range of 100.35 percent. According to ICH guidelines, a low-cost, reliable, precise, linear, and rapid RP-HPLC method was developed and validated. This approach has been shown to be accurate, and it can now be used to analyse Human Insulin on a regular basis.

Keywords: Human Insulin, RP-HPLC, Diabetes, Degradation, Mobile phase, Isocratic

INTRODUCTION

Insulin is a drug that is used to treat diabetes mellitus and hyperglycemia caused by a number of factors. It belongs to the insulin class of drugs known as short-acting insulin. This activity covers the mechanism of action, adverse effect profile, labelled and off-labelled indications, contra-indications, tracking, and toxicity of daily insulin, all of which are essential for members of the healthcare team managing patients with diabetes and associated conditions **[1]**. Human insulin is made up of two peptide chains, A and B, each of which contains 21 and 30 amino acids and is linked together by three disulfide linkages **(Figure 1)**. Evaluation of immunoassays for the measurement of insulin and C-peptide as indirect biomarkers of insulin misuse in sport: values in a selected population of athletes **[2]**, determination of Insulin in humans with Insulin-Dependent diabetes mellitus patients by HPLC with diode array detection **[3]**. evaluation of a Chemi-luminescent Immunoassay for Measure-ment of Equine Insulin **[4, 5]**, quantification of insulin in humans with Insulin-Dependent diabetes mellitus patients by HP To the best of our knowledge **[6, 7]** no method for measuring insulin R quantitatively using RP-HPLC has been published. As a result, the aim of this study is to use the RP-HPLC method to evaluate the insulin R and its degradation product desamido (preservatives in commercial insulin) **[8, 9]**.

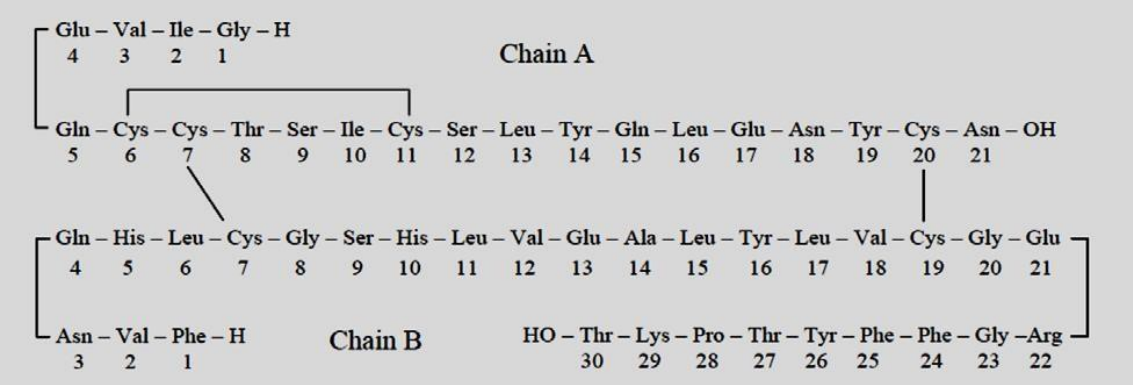

Figure 1: Insulin Structure

METHODS AND MATERIALS

Drugs and reagents:

Human insulin was obtained from Sigma-Aldrich, US (raw material) and Huminsulin R formulation was purchased from local pharmacy. The central raw material store of Regenix Biosciences Ltd provided methanol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), sodium sulphate anhydrous (analytical

grade), ortho phosphoric acid solution (analytical grade), and ethanolamine (analytical grade).

Instrumentation

Analysis were performed on HPLC - 2030 plus-prominence I series (Shimadzu Corporation, Japan) consisting of pump with UV Detector. Separations were achieved on Zorbax 300SB-C_{18}, (4.6 X 250 mm, 5µm particle size) and Lab solution software was used for data collection and analysis. The SHIMADZU AUX-120 analytical balance was used to perform all of the weighing operations for this study. ENERTECH Electronics Pvt. Ltd., India, was used an ultrasonicator to ultrasonicate the samples.

Preparation of Buffer (solution A):

About 56.8g of anhydrous sodium sulfate accurately weighed and transferred in to 2000 ml volumetric flask. Then dissolved it by adding water and made up to the volume with water. About 5.4 ml of phosphoric acid was pipetted out into the solution and adjust with ethanolamine to a pH of 2.3, if necessary.

Preparing the standard solution entails the following steps:

Around 10 mg of Human Insulin standard was correctly weighed and transferred to a 10 ml standard flask, where it was dissolved in 0.01M hydrochloric acid, diluted to the mark with 0.01M hydrochloric acid, and thoroughly mixed (1000g/ml).

Sample solution preparation (Assay of Insulin R):

About 10 pool vials of sample were transfer the content in to clean glass beaker and mix well. 10.0 ml (10 mg) of pooled sample solution was pipette out and transferred to a 10 ml volumetric flask, to which 40 l of 0.01M hydrochloric acid was applied to see whether a suspension could be made visible. To obtain a homogeneous sample from a suspension, shake the specimen before sampling. Small aliquots of 0.01M hydrochloric acid solution were applied if a suspension did not turn clear after 5 minutes of initial acid addition. Until there was a clear solution obtained.

Chromatographic Conditions Optimization

Conditions of Chromatography **(Table 1)** shows the results of optimization.

Table 1: Chromatographic Conditions Optimization

Mode of separation	**Isocratic**
Stationary phase	**Zorbax 300SB-C_{18}, 4.6 X 250 mm, 5µm or equivalent**
Detector wavelength	**214 nm**
Mobile phase	**Solution A: Acetonitrile (74:26 %v/v)**
Flowrate	**1ml/min**
Temperature	**40°C**
Sample load	**10 µl**

Validation of the method

According to the recommendations of the International Conference on Harmonisation (ICH) guidelines, the optimised approach was validated for linearity, precision, LOD, LOQ, and robustness **[10]**.

System suitability study:

System suitability tests are an important component of liquid chromatographic methods, according to the United States Pharmacopeia. For standard solutions, we calculated retention time, area, and the number of theoretical plates. Six duplicate injections of 50g/ml human insulin solution were administered into a device, and chromatograms were taken.

Specificity:

The ability to evaluate the analyte unequivocally in the presence of components that may be present is known as specificity. Impurities, degradants, matrix, and other substances are common examples. Blank, standard, and sample solutions (50g/ml) were prepared. The chromatograms were registered after 10l solutions were injected into the device.

Linearity:

The linearity solutions were prepared 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 and transferring these solutions into six separate 10 ml volumetric flasks and diluting to 10 ml with diluents to obtain 40 g/ml, 45 g/ml, 50 g/ml, 55 g/ml, and 60 The chromatograms were recorded at 214 nm after all of the solutions were injected. The concentration spectrum shown above is linear and follows Beer's law. The calibration was built by plotting the peak areas against concentration.

Accuracy:

The method's accuracy was checked by spiking the drug norm in a predetermined tablet solution at concentrations of 75, 100, and 125 percent and calculating percent recovery studies.

Robustness:

The ability of a system to remain unaffected by minor or intentional changes in chromatographic conditions such as organic content in mobile phase ratio (10) flow rate (0.1), and temperature (one degree Celsius) is referred to as robustness. 10 l of solution was injected into the chromatographic system for each condition, and chromatograms were registered. The effects of the device suitability parameters were examined.

RESULTS AND DISCUSSION

System suitability study:

The values obtained from the device suitability analysis for retention time and peak area were found to be within reasonable limits. **(Table 2)** displays the results.

Specificity:

In the specificity study, no interference was observed from diluents, all known Impurities at the retention time of Insulin peak. The chromatogram was shown in **(Figure 2)**.

Linearity:

The graph between peak areas obtained versus concentrations was plotted to determine linearity and create a calibration curve. For the concentration range of 40-60 g/ml, a linear relationship was found with a slope of 77154, an intercept of 72054, and a correlation coefficient of r2 = 0.9994. y = 777154X + 72054 was the regression equation obtained during the linearity determination. Calibration curve was shown in **(Figure 3)**. The reports of analysis were shown in **(Table 3)**.

Accuracy:

When a known volume of standard drug was applied to pre-analyzed samples and subjected to the proposed HPLC process, the accuracy of the established method was assessed in terms of percent recovery studies at three different levels 75 percent, 100 percent, and 125 percent; percentage of drug recovered. The average recovery percentage was found to be 100.35 percent. 0.4549 was found to be the percent RSD. The RSD value in percent was found to be less than 2%. It proved that the approach was right. **(Table 4)** displays the reports.

Robustness:

The robustness study revealed that minor variations in flow rate, wavelength, and temperature had no effect on the factors chosen. The retention period was discovered to be normal and representative. As a result, the process was reliable. The robustness values are shown in **(Table 5)**.

Table 2: System suitability Study

Parameters	Insulin	Desamido
Retention time	22.435	28.835
Peak area	48148515	367394

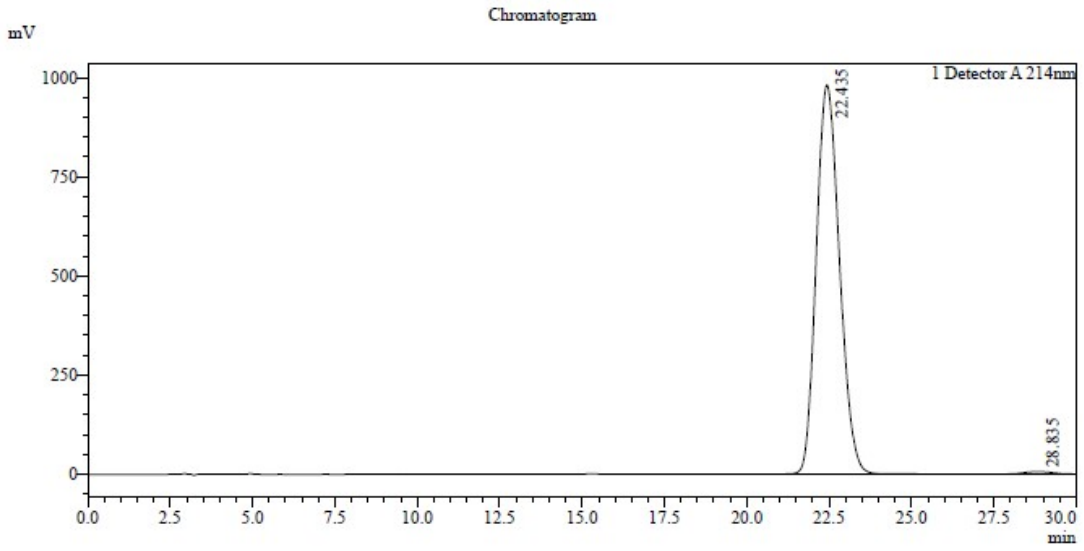

Figure 2: Chromatogram for specificity

Table 3: Linearity Data for Insulin R

Concentration (µg/mL)	Peak Area for Insulin
40	31347399
45	35095557
50	38907733
55	42812869
60	46557328
r^2	0.9994
Slope (c)	72054
Intercept (m)	777154

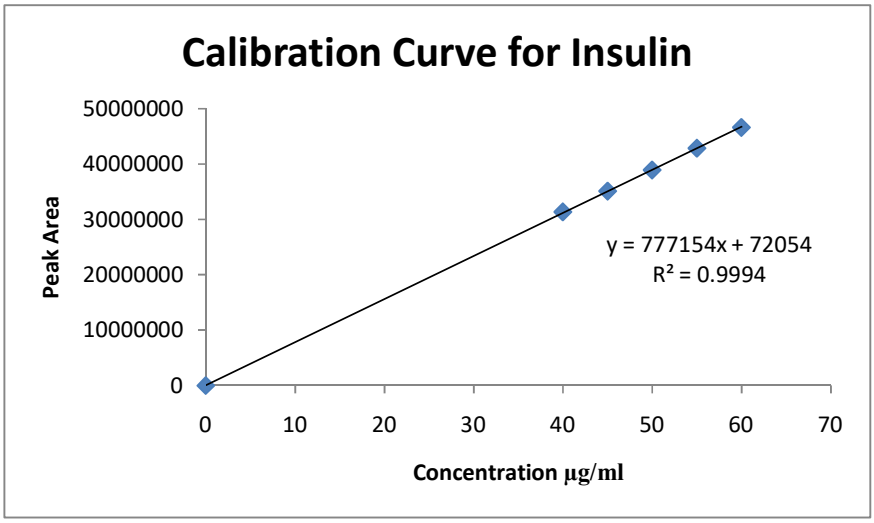

Figure 3: Calibration Curve for Insulin

Table 4: Accuracy Study

Concentration (%)	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovered	Mean	SD	% RSD
75	33.75	33.79	100.11	100.35	0.4565	0.4549
100	45.00	45.40	100.88			
125	56.25	56.29	100.07			

Table 5: Robustness Study

PARAMETERS	CONDITIONS	RETENTION TIME
Low flow rate (ml/min)	0.9 ml/min	22.4
High flow rate (ml/min)	1.1 ml/min	19.0
Low Wavelength	213 nm	20.7
High Wavelength	215 nm	20.9
Low column temperature	39^0C	19.7
High column temperature	41^0C	22.6

CONCLUSION

For the determination of Insulin R, a simple analytical, robust, and isocratic RP-HPLC method was developed. The validation of the developed analytical method yielded results that were within the ICH guidelines' limits. Insulin R was separated using a validated stability-indicating RP-HPLC method capable of separating insulin and its desamido degradation **product.**

CONFLICT OF INTETEREST

There are no conflicts of interest declared by the writers.

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