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Estimation of biphasic isophane insulin 30/70 by using validated RP-HPLC technique

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Abstract

A rapid, specific, reliable, and repeatable RP-HPLC method for estimating biphasic isophane insulin developed. A pH 2.3 sodium sulphate anhydrous buffer is used as mobile phase-A and acetonitrile (76:26) is used as mobile phase-B in the most current verified isocratic RP-HPLC analytical method. The samples were separated using a Zorbax C18 (4.6 x 250 mm), 3.5 m column at a 1.0 ml/min flow rate. The recommended wavelength for ultraviolet detection was discovered to be 214 nm. This technique was evaluated for the investigation of biphasic isophane insulin and its desamido the degradation product in the form of phenol and m-cresol, which are present in small amounts as preservatives in commercial preparations of biphasic isophane insulin. The technique was discovered to be linear over the concentration range of 80-120% with coefficient regression r^2 = 0.9995. The mean recovery was determined to be in the range of 99.40% during accuracy tests. A rapid, accurate, linear, low-cost, and reliable RP-HPLC technology is designed and validated in compliance with ICH standards. Since it has been demonstrated to be reliable, this method can now be regularly used to evaluate biphasic isophane insulin.

1. Introduction

Diabetes mellitus and elevated glucose levels are conditions that are treated with the medication insulin. Biphasic isophane insulin is composed of 70% isophane and 30% human insulin (a combination of short-acting and intermediate-acting). It is a member of the Premixed insulin medication class, which is related to insulin (Taylor *et al.,* 2016; Wahl *et al.,* 2019; Evans *et al.*, 2011; Bruno Sarmento *et al*., 2006; Baynest., 2015).

Diabetes is managed by administering human insulin, a 2-chain peptide that comes from the pancreas of the living being. It is a type of short-acting insulin produced utilizing a recombinant DNA manufacturing process. Intermediate-acting insulin is available in the insulin isophane injectable form, it is a protamine sulphate or another acceptable protamine complexed with bovine, porcine, or human insulin in a sterile suspension. This activity covers the daily insulin's mode of action, side effect profile, labelled and off-labelled indications, contraindications, tracking, and toxicity, all of which are important for individuals on the medical team who are managing patients with diabetes and associated comorbidities (Bilal Yilmaz *et al.,* 2012; Chaluvaraju *et al*., 2012; Morello., 2011).

Biphasic isophane insulin, a dual-acting hormone, promotes glucose uptake while lowering blood sugar (glucose) levels (Divya Singh *et al*., 2021; Mounika *et al*., 2021; Duraisami *et al*., 2021; Venkatachalam *et al*., 2021). It is followed by insulin attaching to adipose and muscle cells, stopping the liver from producing glucose. As a result, biphasic isophane insulin cooperates to reduce blood sugar levels after meals. The chemical has the molecular formula $C_{257}H_{383}N_{65}O_{77}S_6$, a molecular weight of 5808 and it is essentially insoluble in anhydrous ethanol and water, but soluble in diluted mineral acids. Its chemical structure is given (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).

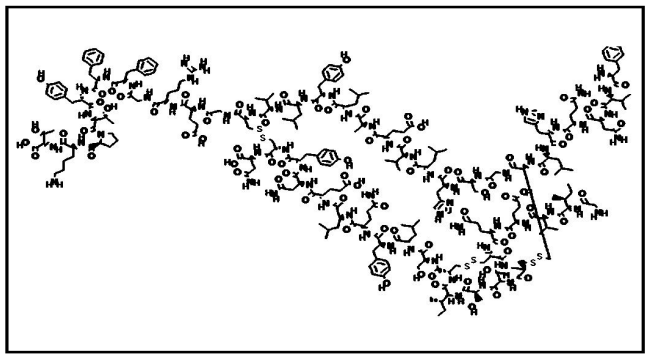

Figure 1: Chemical structure of insulin.

There are not many methods available for determining insulin and its analogues, and those that are available have limitations such as greater operational costs, unfavourable concentration sensitivity, and low sample throughput to the best of our knowledge. There is not reported technique for quantitatively assessing biphasic isophane insulin with RP-HPLC. This study aims to assess biphasic isophane insulin using the RP-HPLC method.

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2. Materials and Methods

2.1 Drugs and reagents

Pharmaceutical aid biphasic isophane insulin 30/70 sample from Regenix Biosciences Ltd. and also offered are sodium sulphate anhydrous (analytical grade), methanol (HPLC grade), acetonitrile (HPLC grade), and water (HPLC grade), orthophosphoric acid solution (analytical grade), hydrochloric acid (analytical grade) and ethanolamine (analytical grade), human insulin standard and porcine standard (working reference standard).

2.2 Instrumentation

Analysis was carried out on HPLC-2030 plus-prominence I series (Shimadzu Corporation, Japan) compromised pumps with UV detector. The Zorbax 300SB - C18 (4.6 x 250 mm, 5 m particle size) was used for separations, and for data collection and processing, lab solution software was employed. The weighing operations for this study were performed on the SHIMADZU AUX-120 analytical balance. The samples were ultrasonically processed using an ultrasonicator from ENERTECH Electronics Pvt. Ltd. in India.

2.3 Mobile phase preparation

Anhydrous sodium sulphate was accurately weighed out at 56.8 g and then added to a 2000 ml volumetric flask. Then, add water to equal the volume after adding water to dissolve it. Phosphoric acid was pipetted into the mixture a total of 5.4 ml, and the pH was adjusted to 2.3 using ethanolamine, if necessary. The buffer and acetonitrile mixture was mixed in a 76:26 (v/v) ratio before being briefly degassed in the ultrasonic water bath. Using a 0.45 filter, the solution was filtered under a vacuum and then used.

2.3.1 Preparation of standard solution A (human insulin standard)

The human insulin standard was precisely weighed at 20 mg and transferred to a 5 ml standard flask where it was thoroughly mixed after being dissolved in 0.01 M hydrochloric acid.

2.3.2 Preparation of standard solution B

10 ml of 0.01 M hydrochloric acid are added to 1 ml of standard solution A.

2.3.3 Preparation of standard solution C (porcine standard)

20 mg of the porcine standard should be weighed into a 5 ml standard flask. The material should be dissolved in 0.01 M hydrochloric acid, then diluted to the desired concentration using 0.01 M hydrochloric acid and thoroughly mixed.

2.4 Preparation of sample solution

Pipette and precisely transfer 10 ml of the combined sample solution into a volumetric flask with a 10 ml capacity. Add 40 µl of 6 M hydrochloric acid to create a clear solution after that, either as a suspension or a solution. To obtain a homogenous sample, shake the material prior to sampling it in suspension. If after the first 5 min of acid addition, a suspension does not turn clear, add small aliquots of 6 M hydrochloric acid (less than 4 µl/ml of test solution) until a definitive conclusion is obtained.

2.5 Chromatographic conditions

The outputs of optimization are given by the conditions of chromatography in Table 1.

Table 1: Chromatographic conditions

Instrument	HPLC
Column name	Zorbax 300SB-C18, 4.6 x 250 mm, 5 µm orequivalent.
Pumping mode	Isocratic
Rate of flow	1.0 ml/min
Detection of wavelength	214 nm
Column oventemperature	40°C
Injection volume	10 µl
Column pressure	400 kgf/cm^2
Sampler cooler	2 to 10ºC

3. Results

3.1 Method validation

According to the most recent ICH recommendations and in order to achieve the goals of specificity, accuracy, linearity, precision, and robustness, the analytical method was improved and verified.

3.1.1 System suitability test

Retention time and peak area values derived from the system suitability study were determined to be within acceptable ranges. The results be seen in Table 2.

Table 2: Study of system suitability

Parameters	Biphasic isophane insulin	Desamido
Retention time	19.333	24.325
peak	39402000	22419

3.1.2 Specificity

The specificity study discovered no diluent interference despite the fact that they were all recognised impurities at the time the peak was retained. It may be seen on the chromatogram.

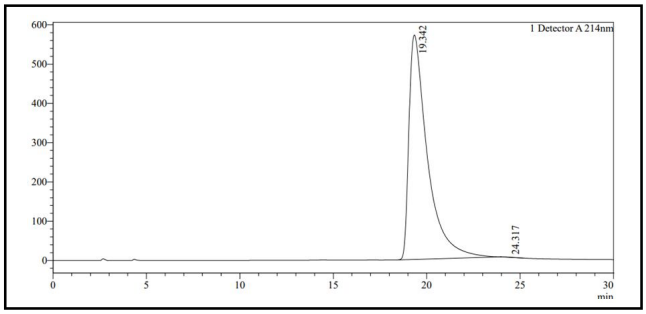

Figure 2: Chromatogram of specificity.

3.1.3 Linearity

In order to evaluate linearity and produce a calibration curve, the graph between the acquired peak areas and concentrations was

generated. With a slope of 2.9002, an intercept of 0.3889, and a correlation coefficient of $r^2 = 0.9995$ for the concentration range of 3.2-4.8 mg/ml, a linear correlation was identified. During the linearity assessment, a regression equation was formed that was y = 0.3889x + 2.9002. Figure 3 displays the calibration curve. Table 3 contains the analysis report results.

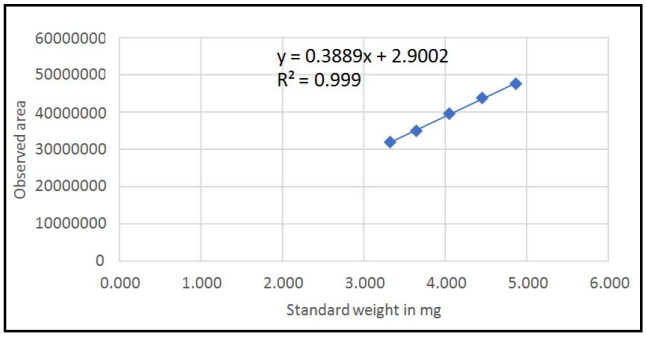

Figure 3: Calibration curve of biphasic isophane insulin.

Table 3: Linearity data

Concentration (%)	Peak area
80	31824738
90	34969382
100	39509721
110	43724424
120	47479633
r^2	0.9995
Slope (C)	2.9002
Intercept (m)	0.3889

3.1.4 Accuracy

At five different levels, the effectiveness of the established procedure was assessed in terms of per cent recovery studies. The amount of drug recovered when a known volume of the reference drug was added to pre-analyzed samples and exposed to the suggested HPLC method ranged from 80% to 120%. It was found that the average recovery rate was 99.40 per cent. RSD as a per centage was measured to be 0.21. The % RSD value was found to be less than 2%. That provided evidence that the method was acceptable. The reports are shown in Table 4.

Table 4: Accuracy data

Concentration (%)	% Recovered	Mean	%RSD
80	99.20		
90	99.50		
100	99.60	99.40%	0.21
110	99.90		
120	99.80		

3.1.5 Precision

Biphasic isophane 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. The reports are shown in Table 5.

Table 5: Precision data method precision

S. No.	Sample ID	Content (% of label claim)
1	Sample-1	101.99 mg (102 %)
2	Sample-2	101.97 mg (102 %)
3	Sample-3	102.08 mg (102.1%)
4	Sample-4	102.15 mg (102.2%)
5	Sample-5	102.20 mg (102.3%)
6	Sample-6	102.26 mg (102.1%)
Mean		102.11 mg (102.1%)
RSD (NMT 2.0%)		0.12%

3.1.6 Robustness

The robustness analysis found that minor modifications in temperature, pH, and flow rate had no impact on the selected parameters. It was found that the retention period was typical and significant. The procedure was trustworthy as a result. The robustness results are given in Table 6.

Table 6: Robustness data

Parameters	Conditions	Retention time
Low flow rate (ml/min)	0.9 ml/min	16.342
High flow rate (ml/min)	1.1 ml/min	18.582
Low pH	1.8	17.533
High pH	2.8	17.600
Low column temperature	39°C	18.550
High column temperature	41°C	16.548

4. Discussion

The United States Pharmacopoeia claims that system suitability studies play a significant role in liquid chromatographic techniques. For standard solutions, we estimated the total number of theoretical plates, the area, and the retention longevity. Chromatograms were collected after injecting a standard solution into the device.

Specificity is the capacity to accurately assess the analyte in the presence of possible constituents. Common examples include impure substances, degradants, frameworks, and other substances. Three solutions-blank, standard, and sample were created. The chromatograms were recorded after solutions had been added to the device.

The normal stock solutions of the sample were divided into six separate 10 ml volumetric flasks, each of which received 0.40, 0.45, 0.50, 0.55, and 0.60 ml of the linearity solutions, which were then diluted to 10 ml with diluents to produce 40 µl/ml, 45 µl/ml,

50 µl/ml, 55 µl/ml, and 60 µl/ml, respectively. Following the injection of each solution, the chromatograms were taken at 214 nm. The concentration spectrum seen above follows Beer's law and is linear. Using a concentration vs. peak area graph, the calibration was produced. The correlation coefficient (R) was found to be 0.9995, which is within the accepted limits.

By adding the drug standard to a specified tablet solution at concentrations of 80, 100, and 120% and calculating per cent recovery studies, the method's accuracy was examined**.** The mean recovery values obtained were 99.40% which indicate that there is extremely less interference coming from matrix components.

Through inter-day precision studies, precision was estimated. Six analyses of the same sample at a concentration of 4 µl/ml were performed on the same day to look for any variations in the results. Inter-day precision three days in a row were dedicated to studying. The % RSD values of inter-day intervals were found to be 0.12%. The results obtained were within the limits.

A system's robustness refers to its capacity to endure subtle or deliberate modifications to the chromatographic conditions, including pH, flow rate (0.1 ml/min), and temperature (1°C). A material solution diluted to a maximum of 10 µl for each condition was added to the chromatographic apparatus.

5. Conclusion

A simple analytical, dependable, and isocratic RP-HPLC method was used to evaluate the presence of biphasic isophane insulin. Outcomes from the evaluation of the new method of analysis were within the ICH guidelines' limits. An established RP-HPLC technique that can distinguish between insulin and its desamido breakdown component was used to separate biphasic isophane insulin**.**

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Conflict of interest

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

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