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METHOD DEVELOPMENT AND VALIDATION OF INSULIN ESTIMATION IN INSULIN DEGRADING ENZYME ASSAY USING RP-HPLC

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ABSTRACT

Insulin Degrading Enzyme (IDE) highly conserved zinc metallopeptidase is the primarily responsible for high specific degradation of insulin. The aim of this project is to develop a simple, sensitive, and rapid chromatographic procedure for the analysis of human insulin remaining in Insulin Degrading Enzyme assay using isocratic RP-HPLC/UV. A column type RP-C18 (150 × 4.6 mm, 5 μm particle size). The eluent consists of 50% sodium sulfate (0.2 M, pH 3.1 adjusted with o-phosphoric acid), 50% ACN. The eluent was pumped at a flow rate of 1.0 mL/min and the effluent was monitored using UV detector at 214 nm. The method produces a linear response over the concentration range of 0.15-25 µg/mL of insulin and linear as well with increase in the protein concentration. The method is validated for specificity, linearity, precision, accuracy and limit of detection and quantification. Also, the method developed can be applied for the estimation of insulin in the IDE assay.

Key words: Human insulin, HPLC, Validation, Isocratic.

INTRODUCTION

Diabetes mellitus is a life-threatening and highly prevalent group of endocrinological disorders, a chronic metabolic disease characterized by the imbalance of blood glucose levels and insulin secretion, causing abnormal glycemic levels. One of the main sources of energy for the body is glucose. Normally, the body maintains blood glucose levels within a narrow range (70-130 mg/dl). The endocrine hormone, insulin regulates the storage of

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glucose it facilitates anabolic metabolism in the body. The insulin secretions are evoked by rise in blood glucose levels by the β- cells of the pancreas[1].

Diabetes is more prevalent in low and middle-income countries. Globally, 366 millions of people suffer with diabetes and it is expects to reach 552 million in 2030. Insulin is the key hormone released by the β-cells of langerhans released into the blood stream owing to rising blood glucose levels. Dipeptidic hormone (A & B chains) with connected together with disulfide bridges. Insulin receptors have high affinity to the insulin, after binding to its receptor it undergoes phosphorylating intracellular target proteins[2]. Insulin stimulates the synthesis of glycolysis, proteins and lipids while lipolysis, gluconeogensis and glycogenolysis is inhibited. The adverse effects of hyperglycemia are hypovolemia, electrolyte imbalance and finally it may lead to coma[3].

Insulin is metabolized primarily in liver and the kidney. Previously, it was anticipated that liver is the main organ responsible for the insulin metabolism, but even kidneys play a crucial role in metabolism. Traces of insulin were observed in the urine. Renal diseases decreased the insulin dose, has confirmed that kidneys play a crucial role in the degradation, secretion and clearance. Exclusion of kidneys from rabbits has prolonged the half-life of insulin. Kidneys are the vital extra hepatic site for insulin metabolism, and may play a central role in the regulating the insulin levels in systemic circulation[4].

Plasma insulin concentration is directly proportional to the excretion rate of insulin. Severe hypoglycemia was observed in subjects after administering oral hypoglycemic drugs in those most of the patients were suffering from the renal and hepatic diseases[4]. It is possible to enhance insulin signaling and availability by inhibiting IDE-mediated insulin catabolism[5].

Insulin Degrading Enzyme (IDE) highly conserved zinc metallopeptidase (EC 3.4.24.56) is the primarily responsible for high specific degradation of insulin, leading to its name[6]. It is ubiquitously expressed, distributed in bacteria, fungi, plants and animals in tissues and at the sub-cellular level. Firstly discovered by Arthur Mirsky in 1949, since then thought that anti-diabetic drugs are acting by inhibiting this enzyme but later on concluded that they act by other mechanisms. Several studies confirm the role of IDE in the insulin degradation like degraded insulin products were observed in the cells and it is IDE inhibitors inhibited the insulin degradation[7].

IDE also known as insulysin, is distributed in most of the mammalian tissues higher amounts in liver, kidney, skeletal muscle, red blood cells etc. Purified IDE has a mass of 110 kDa, reported to exist in equilibrium mixture of monomers, dimers and tetramers and

dimer being the predominant[8]. IDE is a zinc metalloprotease (clan ME, family M16)[8,9]. These protease have the zinc in the catalytic centre and zinc binding site is involved in catalysis, HxxEH. Members of this family are called ‘‘inverzincins’’ because they feature a zinc-binding motif, is an inversion of the sequence found as compared to classical metallo-proteinases HExxH. The two histidines coordinate the binding of the zinc atom and the glutamate acts as a general base for catalysis. This Mutation in these any of these conserved amino acids inactivates protease[9,10].

IDE is distributed in cellular and sub-cellular fractions like plasma membranes, peroxisomes and endosomes[7]. IDE also known to degrade several other peptides other than insulin like Amyloid β peptide¸ Amylin, Glucagon, Calcitonin, Tumour necrosis factor (TNF–α), Atrial natriuretic peptide(ANP), bradykinin, β-endorphin, Insulin like Growth Factor (IGF) I & II[9-12].

Separation of insulin and the degradation products is a difficult mission due to the complex structures. Various immune and non-immune methods have been developed like radio immune assay, enzyme immunoassay, luminescent immune assay, capillary electrophoresis and High Performance Liquid Chromatography for both *in vivo* and *in vitro*.

Many researchers have already reported the determination of remaining insulin in the IDE assay using HPLC but the methods had failed to achieve inadequate resolution and poor chromatographic conditions. Most of the researchers applied the gradient method for estimating the insulin in the IDE assay, which is considered as time consuming because of high retention time.

This paper is aimed to develop a rapid and effective chromatographic procedure for estimating the remaining insulin in the assay system using isocratic RP-HPLC method. The developed method provides simplicity, sensitivity, selectivity, precision and accuracy in the determination of insulin the IDE assay system.

EXPERIMENTAL

Chemicals and reagents

Human insulin was purchased from the Pharmacy (huminsulin) from Eli Lily (Hyderabad, India). Acetonitrile-HPLC grade from Finar (Ahmedabad, India), hydrochloric acid and acetic acid from Rankem (New Delhi, India), o-phosphoric acid 85% from Universal (Mumbai/India), sodium sulfate from Rankem (New Delhi, India), BSA, Tris

hydrochloride and sodium chloride from Himedia (New Delhi). All the other chemicals used were of analytical grade unless otherwise stated.

Instrumentation

The HPLC system consisted of a Shimadzu UFLC Prominence DAD detector (SPD-M20A) equipped with a 1-valve sample injection port fitted with a 20 μL sample loop.

Method development

The following chromatographic conditions were adopted:

Column: Eclipse RP-C18, 150 × 4.6 mm, 5 μm particle size from Agilent (Santa Clara USA);

Detector: Diode Array Detector model SPD-M20A (Prominence-DAD Detector)

Wavelength: 214 nm;

Sample injection loop: 20 μL;

Flow rate: 1.0 mL/min;

Elution mode: isocratic;

Mobile phase: 50% Na_2SO_4 buffer (0.2M. pH 3.1 adjusted with Orthophosphoric acid), 50% ACN, the final pH was adjusted to 3.6. Sodium sulfate buffer is filtered through the 0.22 µm membrane filter

Preparation of insulin stock solution (I)

28 IU (1000 µg) of insulin was dissolved in 10 mL of mobile phase solution. This will give an insulin solution with the concentration of (100 µg/mL) was further diluted by addition of 0.01 M HCl to give required concentrations. The solution were kept in refrigerator (< 5^0C) for not more than two days.

Preparation of standard insulin solutions for the calibration curve

Standard stock solutions of insulin (0.15, 0.30, 0.75, 1.5, 3.0, 5.0, 10, 15, 25) µg/mL diluted with 0.01 M HCl and were injected.

Test animals

The experimental protocol was approved by the institutional animal ethics committee of KLEU`s College of Pharmacy, Belagavi, which was registered with Committee for the purpose of control and supervision of experiments on animal (CPCSEA),

Govt. of India (registration no. 221/CPCS EA with resolution number no KLECOP/IAEC/ Res 18-19/05/2014). Wistar rats of either sex were maintained under controlled conditions for all sets of experiments. The rats were allowed to take standard laboratory feed and water ad libitum. The ethical guidelines for the investigation of the animals used in experiment were followed in all the tests.

Partial purification of IDE from the rat kidneys

6 Wistar rats weighing between (180-200 gms) were sacrificed by the decapitation method. The kidneys were removed and rinsed in the ice-cold 0.33 M sucrose, homogenized in the glass homogenizer immersed in the ice. The homogenate was diluted in 0.33 M sucrose to a ratio of 5 mL/g of tissue. The homogenate was centrifuged at 1,00,000*g/L hr/4^{o}C (Thermo scientific MX 150) high speed ultracentrifuge. Supernatant was measured and it was considered as crude homogenate. 0.21 gms of ammonium sulfate was added per ml of the crude homogenate (30% saturation). Then, the solution was centrifuged at (20,000*g/20 mins /4^{o}C) in Kubato centrifuge. Ammonium sulfate was added slowly by keeping on ice. The above procedure was repeated once again (60% saturation). The precipitate was collected by adding minimum quantity of 20 mM of acetate buffer pH 6.5 to reconstitute. The suspension was dialyzed in above buffer for 3 hrs with frequent changes at 4^{o}C. The suspension was lyophillized and then stored in -80^{o}C for further use. Protein estimation was determined by Lowry method using bovine serum albumin as standard. Presence of IDE is confirmed by the SDS-PAGE by a presence of thick band at 110 KDa.

Preparation of the stock IDE

The partially purified IDE was diluted to 10 mg/mL with the 0.1 M phosphate buffer (pH 7.4) on the day of the experiment.

Incubation of insulin with, IDE (IDE assay)

The stability of insulin was studied by incubating the insulin in the partially purified IDE prepared from the kidneys, which had been incubated with the insulin at 37^{o}C for 30 mins. The reaction was terminated by adding the 50% acetic acid to the reaction mixture. The volume of the system was maintained for 1 mL with 0.1 M phosphate buffer (pH 7.4). The resulting mixture was centrifuged at 10,000 rpm/4^{o}C/ 15 mins. This centrifugation was done to ensure to remove the precipitated proteins, then the supernatant was analyzed for the residual insulin.

Estimation of insulin

Insulin was analyzed by reversed phase UPLC on C18 column (250*4.5 mm, 5 µm).

The mobile phases were acetonitrile and 0.2 M sodium sulfate in 50:50, the pH was adjusted to 3.1 with orthophosphoric acid. The mobile phase was run at 1 mL/min. The UV detector was set at 214 nm.

Enzyme activity with protein concentration

From the stock 0, 0.2, 0.5, 1, 2, 4 mg/mL of the partially purified IDE was taken and incubated with insulin (10 µg/mL), final volume was adjusted to 1 mL with phosphate buffer. After the IDE assay, the supernatants were estimated for the remaining insulin by the RP-HPLC. The three QC protein samples (0.2, 1, 2 mg/mL) were prepared from the stock solution.

Time course of degradation of insulin

IDE (1 mg/mL) and insulin (10 µg/mL) was pre-incubated and at various time intervals and the reaction was terminated by adding the 50% acetic acid. The resulting mixture was then centrifuged at 10,000 rpm/4^{o}C/15 mins, the supernatant was estimated for the remaining insulin by RP-HPLC.

Method validation experiments

1. Selectivity and interference of additives: Selectivity of the method was studies by processing the blank solutions of 0.01 M HCl, blank IDE, vial additives (phenol, m-cresol). Blank IDE Chromatogram are depicted in the Fig. 2.

2. Linear range: The linear range is very important to clarify the concentration range where the relation between the concentrations and signals is linear. From the development experiments, it was shown that the method is linear up to insulin concentration 25 µg/mL in 0.01 M HCl.

Enzyme (IDE) activity is linear with the increase in the concentration of the protein. In order to validate this assumption, four sets of calibration IDE assays were performed containing the 6 different concentrations (0, 0.2, 0.5, 1, 2, 4 mg/mL) and IDE assay is performed. These solutions were injected in duplicate. The peak areas insulin were plotted against the nominal concentrations of the insulin.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated based on the signal to noise ratio. A Signal to noise ratio (S/N) of three is generally accepted for estimating LOD and signal- to- noise ratio of ten is used for estimating LOQ.

Precision

IDE assays were performed with the three QC samples (0.2, 1, 2 mg/mL) of the IDE assay systems were incubated in the buffer system. After the termination and centrifugation, the supernatants were injected twice. Precision of the assay was determined by repeatability (intra day) and intermediate day (inter day). Same laboratory conditions are maintained for assaying the QC samples. The averages were calculated and percentage of remaining insulin was calculated. These back calculated concentrations were subjected to precision calculations (standard deviations and RSD%). All these data are shown in Table 2.

Accuracy

QC samples were chosen for the accuracy in the IDE assay. Each of these solutions was injected three times and the average was calculated. The back calculated concentration for these assay systems was evaluated form the slope and the intercept of the calibration curve. The average, standard deviation, relative standard deviation (RSD%), and relative error were calculated for each concentrations and shown in the Table 3. These values to illustrate the accuracy of the method.

Stability

The stability was evaluated for stability of the remaining insulin in the assay system during the analysis time and storage period. Insulin was analyzed immediately after the termination of the reaction as well as after the 12, 24, 48 hr in the room temperature, 4^{o}C and -20^{o}C for the concentrations of the IDE assay. Stability was calculated as the percent ratio of the concentrations determined after each storage time with respect to the fresh assay. Results are presented in Table 2.

System suitability

The system suitability was assessed by the six replicate analysis of the IDE assay system (100 µL). The acceptance criterion was ± 2% for the percent relative standard deviation (% RSD) for the peak area and retention time for the remaining insulin in the assay system. The efficacy of the column, as expressed by the number of theoretical plates for the six replicate injections was 3632 ± 2.51% (mean ± %RSD) and the tailing factor was 1.59 ± 0.5% (mean ± %RSD).

RESULTS AND DISCUSSION

The method is very selective; there were no interferances by the 0.01 M HCl, phenol, cresol. Fig. 1 represents that there was no interferance peak by the partially purified

protein. Linearity of the method is demonstrated by the standard solutions chromatograms represented in the Table 1. Linearity is confirmed by the corelation coefficient of 0.999 over the range of 0.15-25 µg/mL; data is listed in the Table 1.

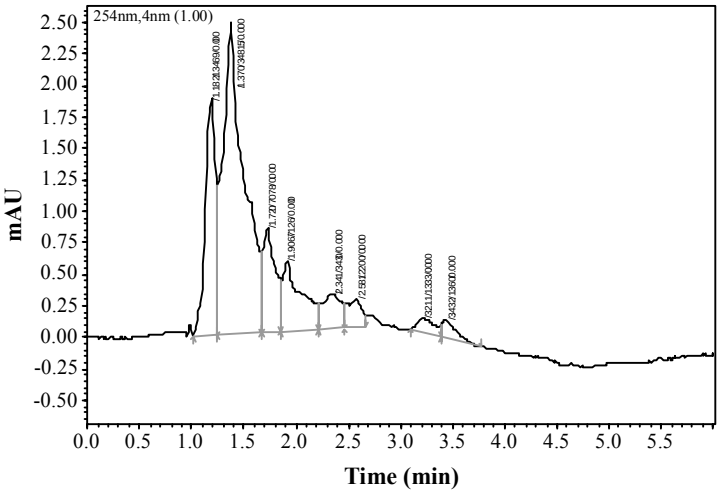

Fig. 1: Selectivity and peak interferences of the chromatogram

LOD and LOQ were assessed for the developed method and found to be 0.063 μg/mL for LOD and 0.211 μg/mL for LOQ.

Table 1: Precision and accuracy of method for estimation of remaining insulin in IDE

IDE (mg/mL)	Inter day		Accuracy	Inter day		Accuracy
	Avg ± SD	%RSD		Avg ± SD	%RSD	
0.2	8.66 ± 0.112	1.29	1.86	8.61 ± 0.30	3.51	3.74
1	3.56 ± 0.08	2.28	2.64	3.75 ± 0.13	3.67	12.89
2	2.04 ± 0.03	1.73	7.84	2.04 ± 0.08	4.28	8.16

SD: Standard deviation of six replicate determinations, RSD:relative standard deviation,

*average of six replicate determinations, Accuracy: (% relative error) (found-added)/ added*100

The precision (repeatbility) was tested at three different concentrations of IDE. Data is represented in Table 2. The RSD values for intra day precision was ≤ 2.28% and for inter day ≤ 4.27%. The accuracy for the intra day ≤ 7.84% and for inter day ≤ 8.16%. These values are summarized in the Table 2.

Table 2: Stability of remaining insulin in the IDE assay

Conc. of IDE (mg/mL)	Room temp. stability recovery % ± SD		Refrigerator stability 4°C Recovery% ± SD		Frozen stability -20°C Recovery% ± SD	
	24 h	72 h	24 h	72 h	24 h	72 h
0.2	96.2 ± 4.56	92.6 ± 6.23	99.4 ± 2.66	96.3 ± 5.22	98.2 ± 4.67	97.2 ± 4.56
1	94.4 ± 2.96	93.9 ± 2.35	99.3 ± 1.77	97.5 ± 4.78	96.5 ± 5.16	98.7 ± 3.67
2	92.3 ± 3.21	88.6 ± 3.69	100.2 ± 2.78	97.4 ± 4.34	99.4 ± 4.21	98.4 ± 2.77

Stability studies were performed for the remaining insulin in the QC samples at room temperature, 4°C and -20°C. Stability was calculated as the percent ratio of the determined concentration after each storage time with respect to the fresh IDE assay. The stability of the results are summarized in the Table 3.

Table 3: System suitability study of remaining insulin in IDE assay

IDE concentration (0.1 mg/mL)	Retention time (t_R)	Peak area
Mean = 6	2.9035	77660
SD	0.0040	2856
% RSD	0.001	3.10

The system suitablity was analyzed by six replicate analysis of the 0.1 mg/mL IDE assay. The results are summarized in the Table 3.

The aim of this work was focused on developing a simple chromatographic method for insulin estimation in the IDE assay without the use of sophisticated and rare gradient elution and without the employing of harsh mobile phase (low pH and highly salinity) or uncommon chemicals and columns. On the same time, the designed method may be applicable for routine analysis of insulin in the IDE assay. This was done by fine-tuning of mobile phase composition and using small particle size for stationary phase.

The peak areas of the remaining insulin in the IDE assay were compared with the standard calibration curve of the insulin. The linearity is checked for both the insulin standard solutions and as well as the enzyme activity with the protein concentrations by calculating the R^2 (Correlation coefficient) value, which is 0.99 for the insulin standard curve where as for the enzymatic activity is 0.73; Fig. 2 depicts the constructed calibration curve for the insulin and Fig. 3 shows the calibration curve for the enzymatic activity. Time course degradation of insulin was depicted in the Fig. 4. The peak is sharp and symmetric represented in the Fig. 5.

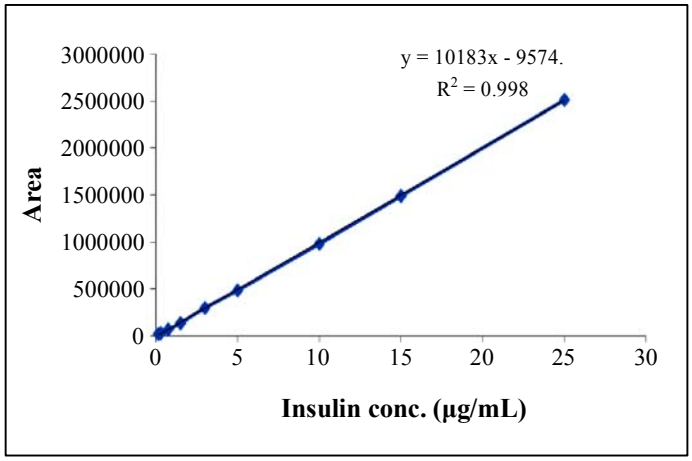

Fig. 2: Calibration curve of standard insulin solution

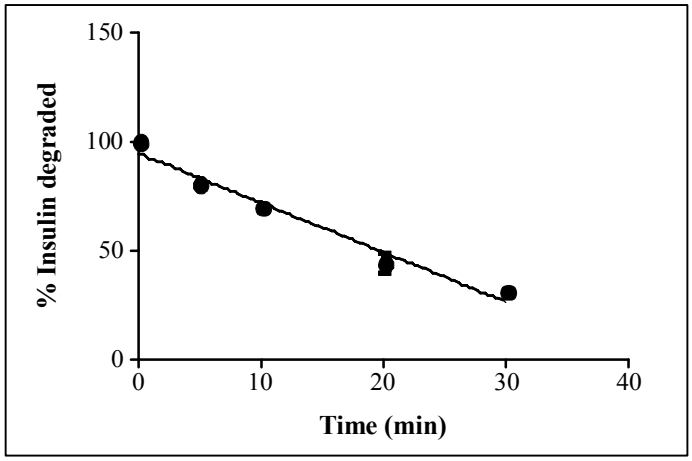

Fig. 3: Time course degradation of insulin in the IDE assay

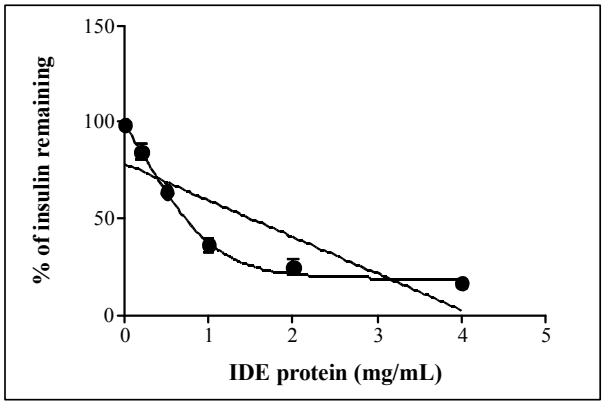

Fig. 4: Percentage of insulin remaining with different concentrations of IDE

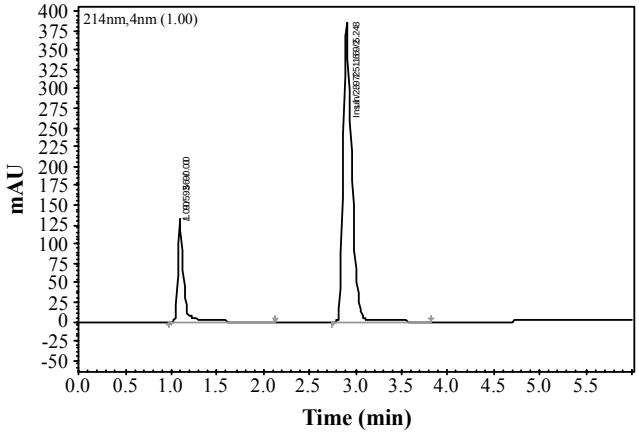

Fig. 5: HPLC UV chromatogram of the standard insulin 25 µg/mL

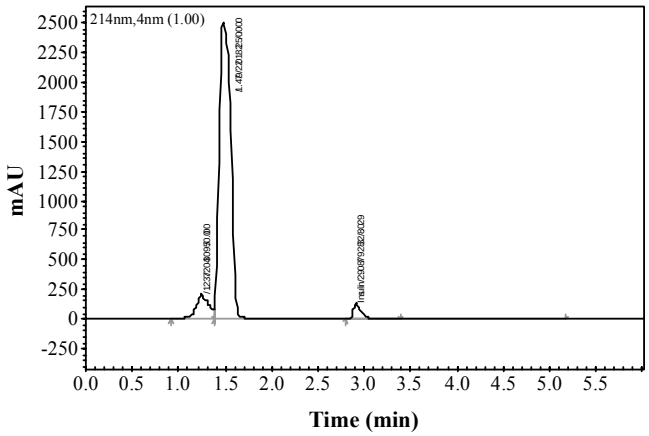

Fig. 6: HPLC-UV chromatogram of insulin in the IDE assay

The present findings was good in agreement with the Duckworth et al. results, who demonstrated that liver and the kidneys are the important organelles that degrades the insulin from the systemic circulation.

Previously, there were many reports using the gradient method for estimation of insulin in the various homogenates, here we report the isocratic method for estimation of insulin. Normally, gradient methods were used for identification of the degraded products, but in the present study we just aimed to quantify the remaining insulin in the assay system so we developed an isocratic method. In takes much time for quantification of insulin in the gradient method, whereas in the present isocratic method it was approximately 3 min.

Khaksu et al. reported that mobile phases with low pH and high salinity produce a good linear shape of the chromatograms. We performed changing the pH (2.5-3.5) and various mobile phase compositions but we observed a significant peak with present mobile phase. An increase in the peak area was observed in proportional to the concentration of the insulin. The selected mobile phase was chosen because of the linearity of human insulin. The developed method gave more sharp and symmetric peaks, even shorter retention time. The method approved its rapidity where the retention time of insulin is 2.9 min.

The selectivity of the method was established by comparing the chromatograms of standard insulin with degraded insulin as shown in Figure 1. The chromatograms proved that no interferences occurred with the retention time (R_t) of insulin peak. The method also shows good resolution.

The precision (repeatability) was tested at three different levels of IDE concentrations. Data presented in Table 2 revealed acceptable values for method precision. Relative standard deviations (RSD%) were ≤ 2.28% for intraday and ≤ 4.27% for inter day. since this is an enzymatic study, variations were observed when compared with the standard precision method.

The accuracy of the method was studied at three different concentration levels. It was 93-100% for intraday and 92-100% for inter day.

CONCLUSION

The new developed isocratic RP-HPLC analytical method for the determination of insulin is simple, sensitive, selective, precise, accurate, and rapid with a short run time of 2.91 min compared to the USP method of 90 min. The method employed a C18 column with ultraviolet detection at 214 nm.

This method was tested for the study of insulin degradation in the IDE. The method proved its validity and therefore can be used for the routine analysis of human insulin degradation in the IDE assay.

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