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Research Article

ELECTROPHORETIC ESTIMATION OF INSULIN: A NOVEL APPROACH TO AN OLDER TECHNIQUE

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ABSTRACT

The objective of the study was to develop a simple and cost effective method for qualitative and quantitative determination of Insulin. In the present study, Insulin was quantitatively estimated Polyacrylamide Gel Electrophoresis (PAGE) and Gel Documentation. PAGE was used as the qualitative tool to develop the bands of insulin. The developed bands were quantified by Gel documentation system. Acrylamide was used as the gelling agent, bis-acrylamide as the cross linking agent, Tris hydrochloride as the separating gel buffer and Coomassie brilliant blue was used as the staining solution in PAGE. A chitosan gel preparation containing 4IU of Insulin per 100µl was optimized and the stability studies for that gel preparation were conducted for 30days in refrigerated temperature using PAGE and Gel Documentation by comparing it with a marketed product. The stability subjected samples in refrigerated condition passed the stability test and the levels of Insulin were in the acceptable limits.

Keywords: Insulin, Electrophoresis, PAGE, Gel documentation.

INTRODUCTION

Insulin is a peptide hormone composed of 51 amino acid residues and has a molecular weight of 5808 Da. It is produced in the Islets of Langerhans in the pancreas. It is a hormone that has extensive effects on metabolism and other body functions. It makes the cells in liver, muscle and fat tissue to take up glucose from the blood, storing it as glycogen in the liver and muscle, and stopping the use of fat as an energy source.

It is used medically to treat some forms of Diabetes Mellitus. Patients with type1 diabetes mellitus depend on external insulin for their survival because the hormone is no longer produced internally. Some patients with type2 diabetes may eventually require insulin when other medications fail to control blood glucose levels adequately.

Pure insulin is known to be very stable and retains its activities for years in a sterile solution (pH 4) at 2^{0}C. The biological activity of crystalline insulin has been found to stay intact for 2 years at 0^{0}C but shows a 29% loss after 1 year at 20-25^{0}C. In diluted acids, insulin is cleaved into several fragments with hydrolysis occurring preferentially at its asparagines and glutamine residues. In alkaline solutions, insulin is rapidly decomposed, resulting from the hydrolysis of its amide linkages and the degradation of the threonine residues.

The various methods of analysis of insulin include Chemical methods, Refractometry method, UV method, Electrophoretic methods, Chromatographic methods, Immunological methods, Turbidimetric and Nephelometric methods

The present study emphasizes the use of electrophoretic techniques like PAGE and Gel documentation in estimating Insulin because of its ability to separate and quantify the molecules based on their size, charge and shape. Electrophoretic techniques were introduced by Tiselius in 1937 whereby proteins were separated in an electrolyte solution within a quartz U-shaped tube through which an electric current was passed. This technique was known as moving boundary or frontal electrophoresis. The term Zone electrophoresis was introduced to refer to the migration of charged macromolecules in a porous supporting medium such as cellulose paper, cellulose acetate sheets, agarose gel film, starch gel and polyacrylamide gel. Zone electrophoresis differs from moving boundary electrophoresis in that it generates an electrophoretogram, a display of protein zones, each one sharply separated from neighbouring zones on the electrophoretic support material. The rate of protein migration is dependent on factors like net electric charge of the molecule, size and shape of the molecule, electric field strength, properties of the supporting medium and the temperature of the operation.

MATERIALS AND METHODS

Materials

Human insulin was a gift from Torrent pharmaceuticals ltd, Ahmedabad, Human Actrapid vial were purchased from a pharmacy, Chitosan (low molecular weight) was kindly given by Indian Fisheries department, Kerala. Acrylamide (AR grade), Bis-acrylamide (A R grade), Coomassie brilliant blue R 250, N,N,N',N'-Tetra methyl ethylene diamine, Tris free base A R grade), Tris hydrochloride (A R grade), Ammonium persulphate (A R grade), Bromophenol blue, Glycine, Potassium dihydrogen orthophosphate, Ethylene diamine tetra acetic acid, Sodium hydroxide (flakes, Sodium chloride were purchased from Himedia chemicals ltd. Glycerol, Ethanol (absolute), Glacial acetic acid and Concentrated hydrochloric acid were purchased from sd-fine chemicals ltd.

Equipment

Electrophoresis system (Vertical mini gel system from bangalore genei), Gel documentation system (GDS-8000 Image acquisition and analysis system from Ultra Violet products), UV Spectrophotometer (Elico instruments), Centrifuge (Remi instruments), pH meter (Global instruments), Micro pipettes (Finn pipettes), Magnetic stirrer (Remi instruments).

Preparation[1] and optimization of the gel

Insulin was dissolved in an aqueous 2% v/v acetic acid solution. To the clear solution chitosan was added gradually and stirred gently for 20 min and it was kept aside for 1hr to allow the polymer to swell. Then the permeation enhancer ethylene diamine tetra acetic acid (EDTA) was added and stirred gently with a glass rod for 10min. Then the preparation was kept under vacuum to remove the air bubbles if any. In order to get an optimized formulation three formulations were prepared with 2, 3 and 4 % w/v of chitosan.

Optimization of the gels was mainly based on viscosity of the formulation and their permeation studies. With 1% of chitosan the prepared gel was found to be very less viscous and there is no three dimensional network. So the optimization was carried out with 2, 3 and 4% chitosan. From the permeation studies 2% chitosan formulations were eliminated, this is due to the rapid release of the entrapped insulin from the gel formulation this may be due to the

insufficient viscosity of the gel. Further formulations were attempted with the 3% chitosan, from the in vitro evaluations it was observed that the drug release was observed for a period 6 hours and also the consistency of the gel formulation was found to be optimum for the nasal administration. Later it was also tried with the 4% chitosan, but the release was extended for a period of 8 hours and also the viscosity of the formulations was very high, which may not be suitable for the nasal administration. Release profile of the optimized formulation was aptly fitted into Higuchi model of drug release with a correlation coefficient value of 0.9770. So the further stability studies were carried out with the 3% chitosan gel formulations.

Stability studies

The stability studies were conducted by Poly Acrylamide Gel Electrophoresis (PAGE) and Gel Documentation techniques. The parameters that were optimized are:

1. Concentration of the acrylamide solution – 7.5%
2. Concentration of the crosslinker i.e. Bis-acrylamide – 0.4%
3. Concentration of the staining solution – 0.25%
4. Time of staining of the gel – 2hr
5. Potential of the DC applied – 100V

The following solutions were prepared to carryout electrophoresis:

1. 30% Acrylamide solution:

Acrylamide - 29.2 g

Bis-acrylamide – 0.8 g

These were dissolved in distilled water and stored in dark bottles at 4^0C.

2. Separating gel buffer (1.5 M Tris hydrochloride of pH-8.8)

Tris hydrochloride – 23.64 g

Distilled water – 75ml

Then the pH was adjusted to 8.8 using sodium hydroxide solution and the volume was made upto 100ml with distilled water.

3. Ammonium persulphate solution (APS) (Initiator)

0.437 g of Ammonium persulphate (APS) was dissolved in 3.1 ml of distilled water.

4. Electrode buffer

3.02 g of Tris freebase and 18.8 g of Glycine were dissolved in 1ltr of distilled water.

5. Sample loading buffer solution

1M Tris hydrochloride (pH-6.8) – 2.5ml

Glycerol – 2ml

1% Bromophenol blue – 1ml

Distilled water – 4.5ml

6. Staining solution

0.25 g of Coomassie brilliant blue was dissolved in 45ml of methanol and 10 ml of glacial acetic acid solution. The volume of this solution was made to 100ml by distilled water.

7. Destaining solution:

Distilled water of about 300ml.

8. Resolving gel (7.5% Acrylamide)

30% Acrylamide solution – 2.5 ml

1.5M Tris hydrochloride solution (pH-8.8) – 2.5 ml

Distilled water – 5ml

Tetra methyl ethylene diamine - 10µl

Ammonium persulphate solution - 100µl.

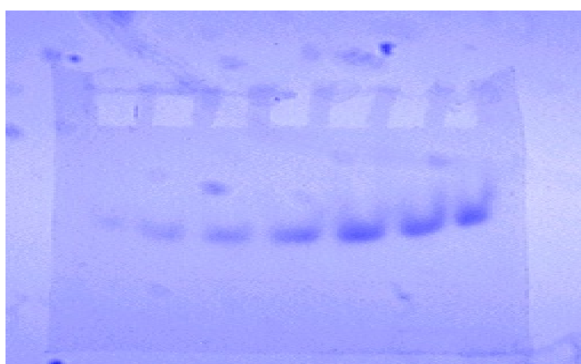
It took about 20min to solidify. So within this time the solution was poured in between the glass plates and the comb was introduced to make the wells. The wells were then loaded with the samples containing 50µl of drug solution and 25µl of sample loading buffer. Then the glass plates containing the loaded gel were placed in the electrophoresis system and the electric potential was applied. It took about 2hr for running of the insulin bands in the gel. The bands of the insulin thus separated by the electrophoresis were quantified by the Gel documentation system.

RESULTS AND DISCUSSION

Standard graph of insulin

Standard graph of insulin was constructed in phosphate buffer (pH-6) by polyacrylamide gel electrophoresis and gel documentation by taking 50µl of solutions containing the amounts of insulin in the range of 10-800µg. The graph was linear with a correlation coefficient of 0.9822. The Integrated optical density is calculated based on the intensity of the band and the area of the band.

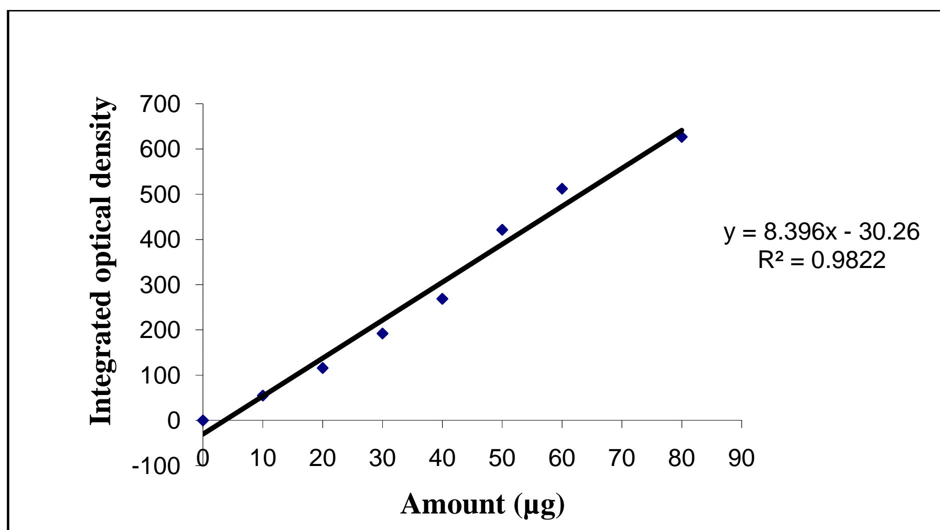
Amt (µg) 10 20 30 40 50 60 80



Table

Amount(µg)	Intrinsic optical density
0	0
10	54.93
20	115.88
30	192.22
40	268.87
50	421.65
60	512.47
80	626.93

Polyacrylamide gel showing the bands and table showing the intrinsic optical densities for the different amounts of Insulin



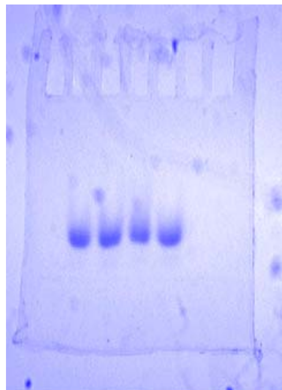
Standard graph of Insulin in phosphate buffer (ph-6)

Stability studies

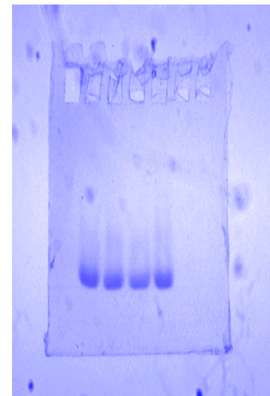
After optimization of the Insulin gel, 10ml of the optimized gel was prepared and it was then divided into 12 equal parts of 0.5ml each. Six time points were taken to assess the stability of the gel. They were 0th day, 5th day, 10th day, 15th day, 20th day and 30th day. 2 parts were used for stability testing at each time points.

Similarly from the marketed product (Human Actrapid) of 10ml, 12 equal parts each of 0.5 ml were divided and for each time point of stability testing, 2 parts were used. All the formulation lots of both test and reference were kept in the refrigerator maintained at a temperature ranging from 2 ^{0}C to 4 ^{0}C.

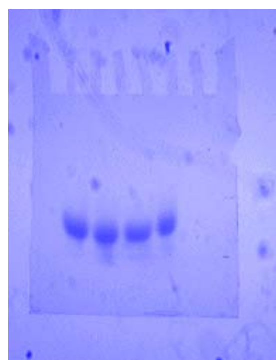
The study was conducted by injecting 50µl of samples into the wells of the polyacrylamide gel and the electrophoresis run was initiated with the different optimized parameters as described in the materials and methods section.



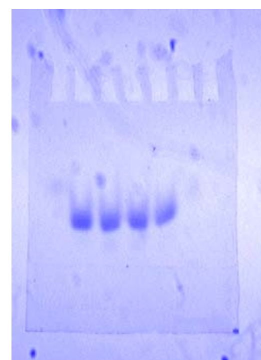
On the day of preparation



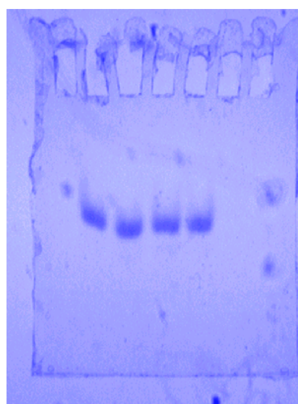
On 5th day



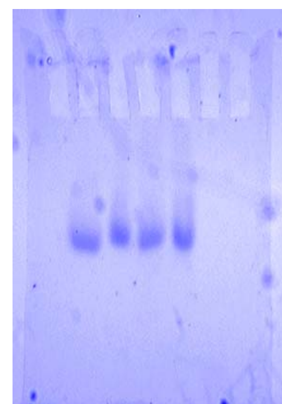
On 10th day



On 15th day



On 20th day



On 30th day

Table showing the Integrated Optical Densities of samples at different time points

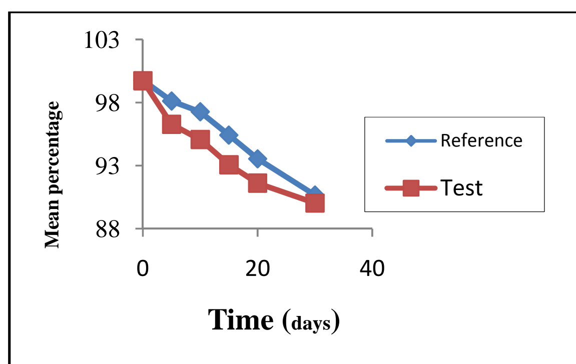
Time (days)	Integrated optical density			
	Reference	Reference	Test	Test
0	557.62	552.92	554.18	556.11
5	544.44	548.47	536.63	534.62
10	542.01	540.91	527.57	529.50
15	534.11	526.98	517.07	516.40
20	519.25	519.76	508.93	507.33
30	505.32	499.69	499.94	497.76

Table showing the Amounts of Insulin in the samples at different time points

Time (days)	Amount (µg)				Mean (µg)	
	Reference	Reference	Test	Test	Reference	Test
0	70.02	69.46	69.61	69.84	69.74	69.73
5	68.45	68.93	67.52	67.28	68.69	67.40
10	68.16	68.03	66.44	66.67	68.09	66.55
15	67.22	66.37	65.19	65.11	66.80	65.15
20	65.45	65.51	64.22	64.03	65.48	64.13
30	63.79	63.12	63.15	62.89	63.46	63.02

(Theoretically calculated amount of Insulin present in the injected volume of 50µL = 70µg)

Table and graph showing the mean percentages of Insulin levels at different time points



Stability of Insulin

Time (days)	Mean percentage (%)	
	Refrigerator	Room
0	99.77	99.75
5	98.12	96.28
10	97.27	95.07
15	95.42	93.07
20	93.54	91.61
30	90.66	90.03

Based on these results, the Insulin gel was found stable under refrigerated conditions for a period of 30days using PAGE and Gel documentation techniques.

CONCLUSION

Based on the above study, PAGE can be used not only as a separation tool as it is being used from long back but also as a quantification method for high molecular weight compounds like proteins if it is accompanied with gel documentation system. One of the applications of this use is the above study and it is also cost effective when compared to that of Chromatographic methods. So it can be an alternate method for assessing the stability of high molecular weight compounds like Insulin.

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