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PHYSICO CHEMICAL CHARACTERIZATION OF INSULINS

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ABSTRACT

The aim of the present work was to develop a physiologically accurate profile of insulin absorption and drug action. Early insulin research methods for its extraction in the purest possible form from animal pancreas tissue and on methods for its large-scale production. The physicochemical characterization of insulin then followed over the subsequent two-and-a-half decades. Milestones included the crystallization of insulin the determination of its molecular weight and the demonstration that it consisted of a pair of disulfide-linked polypeptide chains, namely the acidic chain A and the basic chain B, illustrated that insulin crystals diffracted X rays, deducing at the same time the likely dimensions of the insulin molecule. The introduction of recombinant DNA technologies has allowed the development of insulin analogs, which, through their more physiologic pharmacokinetic profiles, have revolutionized insulin therapy. By using the physicochemical characterization of insulins we will determine its onset of action, duration of action, storage conditions. Among the different analogues produces by insulin we can determine which are rapid acting, long acting, intermediate acting analogues of insulin. Variations in physical activity also modify the glycemic effects of a particular dose of insulin. However, the greatest sources of variation in insulin action are frequently the differences in the physicochemical properties of insulin that affect its diffusion and its absorption in the subcutaneous tissue

Key words: Insulin, Physicochemical characterization, X rays, Recombinant DNA technologies.

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INTRODUCTION

Insulin is a peptide hormone produced by beta cells of the pancreas, and is central for carbohydrate and fat metabolism in the body. It causes cells in the liver, skeletal muscles, and fat tissue to absorb glucose from the blood (1). Insulin stops the use of fat as an energy source by inhibiting the release of glucagon. Except in the presence of the metabolic disorder diabetes mellitus and metabolic syndrome, insulin is provided within the body in a constant proportion to remove excess glucose from the blood, which otherwise would be toxic. When blood glucose

levels fall below a certain level, the body begins to use stored sugar as an energy source through glycogenolysis, which breaks down the glycogen stored in the liver and muscles into glucose, which can then be utilized as an energy source (2). As a central metabolic control mechanism, its status is also used as a control signal to other body systems (such as amino acid uptake by body cells). In addition, it has several other anabolic effects throughout the body. Porcine insulin is especially close to the human version. The primary structure of bovine insulin was first determined by Frederick Sanger in 1951 (3-6). After that, this polypeptide was synthesized independently by several groups. The 3-dimensional structure of insulin was determined by x-ray crystallography in Dorothy Hodgkins laboratory in 1969 (7). The amino acid sequence of insulin is strongly conserved. Bovine insulin differs from human in only three amino acid residues, and porcine insulin in one.

Human insulin

The amino acid sequence of human insulin was published in 1960. The structure was first confirmed in 1982 by x-ray crystallography, in which complete overlaps of x-ray diffraction patterns were achieved on exposures of insulin from human pancreas and human insulin prepared from porcine insulin. Additional complementary identity tests like HPLC and immunochemical cross-reactivity with anti-insulin sera have substantiated the identity of insulin from human pancreas with human insulin prepared from porcine insulin. Human insulin (Novo) has been prepared from crude porcine insulin by intertwining the chromatographic purification processes for making monocomponent porcine and bovine insulin with two chemical reactions; a trypsin-catalyzed transpeptidation reaction and a non-enzymatic cleavage of an ester bond.[21] The human insulin thus obtained complied with the purity specifications of the monocomponent porcine and bovine insulins. The physico-chemical properties of human insulin are similar to those of porcine insulin; hence the analogous preparations for therapy (Actrapid, Monotard, and Protaphane) can be made. Human insulin shares the biologic characteristics of the other mono component insulin's.

Humulin was the first medication produced using modern genetic engineering techniques in which actual human DNA is inserted into a host cell (*E. coli* in this case). The host cells are then allowed to grow and reproduce normally, and due to the inserted human DNA, they produce a synthetic version of human insulin. One distinguishes human insulin's and their analogues like insulin lispro, insulin aspart, insulin glargine which differ from it by certain amino acid and have different kinetics of action (8-11).Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) is a widely used and well-established tool for the analysis and purification of biomolecules. The reason for the central role that RP-HPLC now plays in analyzing and purifying proteins and peptides is Resolution: RP-HPLC is able to separate polypeptides of nearly identical sequences, not only for small peptides such as those obtained through trypsin digestion, but even for much larger proteins. Polypeptides which differ by a single amino acid residue can often be separated by RP-HPLC. In this method, the hydrophobic interactions between the column packing stationary phase and the hydrophobic regions of the protein are exploited.The main objective of this work is to determine the physicochemical characterization of insulin and its analogues according to BP and USP guidelines.

Physico Chemical Characterization of Insulins (12)

p^H Meter Operation

Transfer about 20-25ml (from pooled sample) into dry beaker of 50ml. Allow sample to attain room temperature. Insert a magnetic stirrer into beaker containing sample and place it on a magnetic stir plate, Stir sample slowly, Dip the PH electrode into sample. Note the of P^H sample at a temperature $25^0c \pm 2^0c$. p^H of insulin is 7.0 (Table-1)

Table-1 pH of various Insulin Analogues

FORMULATION	Glargine	Insugen-R	Insugen-N	Insugen-30/70	Insugen-50/50
40IU/ml	4.154	4.022	7.411	7.308	7.337
100IU/ml	4.033	7.614	7.406	7.394	7.420

Determination of Assay of Insulin using RP-HPLC

Preparation of Standards and Samples

4.0 mg/ml of Standard and Sample prepared using 0.01 M HCL. Inject resolution solution and reference solution (d). Record chromatogram of resolution solution until peak corresponding to principle peak in chromatogram obtained with reference solution (d) is clearly visible.In chromatograph obtained with resolution solution identify peaks due to porcine and human .the test is not valid until resolution between peaks due to porcine and human is atleast 1.2. If necessary adjust acetonitrile concentration in mobile phase until resolution is obtained. Inject test solution and 20µl of reference solution (a) and (e) for insulin preparations containing 10IU/ml (or) 20µl of solution(b) and (f) for insulin preparations containing 40IU/ml. if necessary make adjustment of mobile phase to ensure the presence of anti-microbial preservative in test solution and well separated from insulin and show shorter retention times. A small reduction in acetonitrile concentration increases retention time of insulin peaks increases preservatives. if necessary after chromatography wash column with acetonitrile and water for sufficient times to ensure elution for next injection.ths test is not valid unless area of principle peak in chromatogram obtained with reference solution (a) or(b) is 10±0.5 times the area of principle peak in chromatograph obtained with (e) and (f).if this test failed adjust injection volume between 10µl and 20µl in order to be in linearity range of detector. Calculate content of insulin and A21Desamido insulin from area of peak due to bovine, porcine (or) human insulin and that of any peak due to insulin and that of any peak due to insulin and A21Desamido human insulin in bovine insulin RS, porcine insulin RS(or)human insulin.

Procedure The specified volume of Blank, Standard, and Test Preparation was injected into the chromatographic Column and the responses were recorded. The average purity was calculated by integrating the main peak.

100units = 3.47mg of human insulin to 3.45mg of porcine insulin and to 3.42mg of bovine insulin (Fig-1, 2).

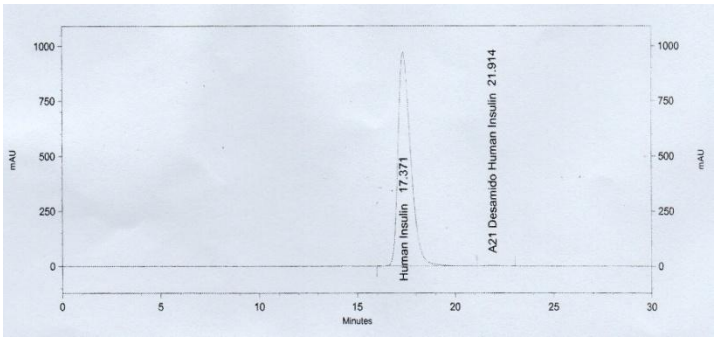

Fig-1 Assay standard chromatogram

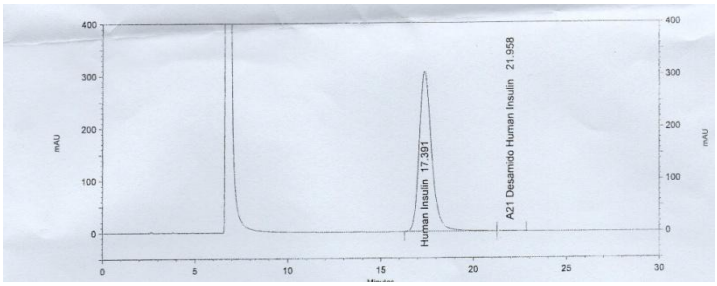

Fig-2 Assay sample

Related proteins

Standard solution

Reference solution a: - weigh and transfer between 36.0mg- 44.0mg of human insulin in 10ml volumetric flask dissolve and make up to the mark with 0.01M HCL to get 4mg/ml.

Reference solution b: - 4ml of reference –a into 10ml volumetric flask and make up with0.01mHcl.

Reference solution d:-4mg/ml of porcine reference standard was prepared by adding 0.01M HCL.

Reference solution f: - 5ml of reference (b) and 5ml of reference (a) into 10ml volumetric flask and make up with0.01mHcl.

Test solution:

1ml of Sample add 4µl of 6M HCL to obtain a clear solution which is injected.

System suitability

Inject 20µl of test solution and 20µl of reference solution (a) for insulin preparations containing 100IU/ml. and reference solution (b) and test solution for insulin preparations containing 40IU/ml. record chromatograph for 15-20 min.

The sum of peaks obtained due to insulin and A21Desaimdo insulin is not more than 6.0% (Fig-3, 4).

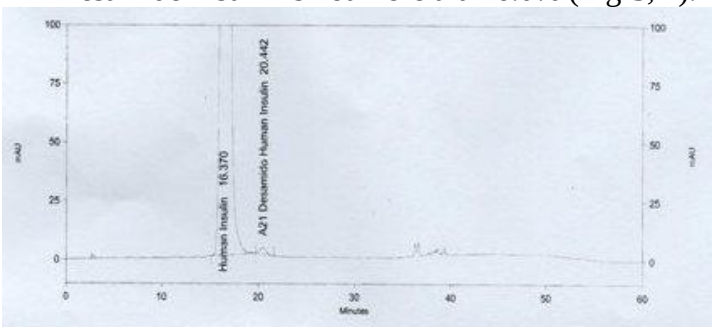

Fig-3 Related Proteins standard chromatogram

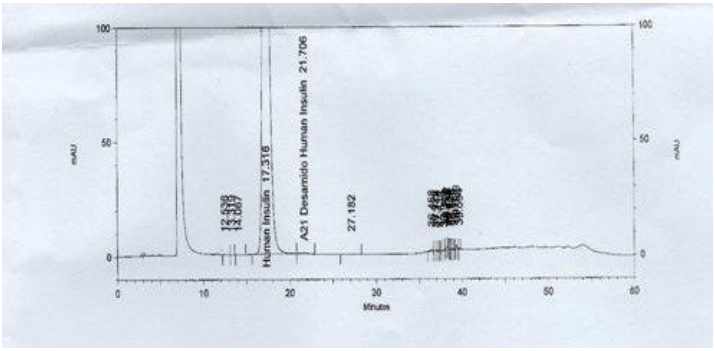

Fig-4 Related proteins sample chromatogram

Content uniformity

4.0 mg/ml of Standard and Sample prepared using 0.01 M HCL. Take 10 individual units de-crimp the lined seal. Push the plunger using glass rod and carefully transfer contents of each cartridge to clean and dry glass test tube individually add 12µl of 6M HCl mix well to obtain clear solution into each test tube allow to stand for 1hour. With draw the sample from each of these 10 individual test tubes into HPLC vial and analyze for uniformity of content. % RSD should be more than 6.0% and each individual assay should not deviate more than 85.0%- 115% of the label claim. If 1 unit is outside the range of 85.0% - 115.0% of label claim and no unit is outside the range of 75.0% - 125.0% of label claim. If RSD is more than 6.0% test 20 additional units.

CONCLUSION

The scope and objective of the present work is to determine the all the physicochemical Properties of the formulations of Insugen 40IU, Insugen 100 IU, Insugen 30/70, Insugen 50/50 are within the specified limits when compared with the standard limits.Short-acting: Includes *regular* insulin which begins working within 30 minutes and is active about 5 to 8 hours.One international unit of insulin (1 IU) is defined as the "biological equivalent" of 34.7 µg pure crystalline insulin. IU/ml stands for international units per milliliter. Insulin is available in two strengths: 40IU/ml, which means that each milliliter contains 40 units of insulin and 100IU/ml which means contains 100units of insulin. An insulin containing 100 units per milliliter is more concentrated than one containing 40 units per milliliter.

100 IU benefits

Less Pain; More comfort – High concentration ensures less volume & thus less pain.Technologicallysuperior– 3rd generation system with recombinant protein expression.Affordable Innovation – Cost of therapy lowered by 30-40% compared to 40 IU.Combination insulin products – Includes a combination of either fast-acting or short-acting insulin with longer acting insulin, typically NPH insulin. The combination products begin to work with the shorter acting insulin (5–15 minutes for fast-acting, and 30 minutes for short acting), and remain active for 16 to 24 hours. There are several variations with different proportions of the mixed insulins. Insugen contains 70% aspart protamine [NPH], and 30% regular insulin).70/30 starts acting fast. If you have type 2 diabetes, inject Mix 70/30 up to 15 minutes before or after starting your meal. If you have type 1 diabetes, inject it up to 15 minutes before you eat a meal. Mix 70/30 is different because it is single insulin that works in two ways. it works fast to cover blood sugar surges at meals, and also has an extended release that can last up to 24 hours.70 will start to lower your blood sugar 10-20 minutes after you take it, a maximum effect occurs between 1 and 4hours and the effect lasts for up to 14-24 hours.Insugen 50/50 [50% insulin lispro protamine suspension and 50% insulin lispro injection, (rDNA origin)] has two phases of absorption. The early phase represents insulin lispro

a rapid-acting blood glucose-lowering agent and its distinct characteristics of rapid onset. The late phase represents the prolonged action of insulin lispro protamine suspension. an intermediate-acting blood glucose lowering agent.Insulin lispro, the rapid-acting component of Insugen 50/50, has been shown to be equipotent to Regular human insulin on a molar basis. One unit of Insugen 50/50 has the same glucose-lowering effect as one unit of Regular human insulin, but its effect is more rapid and of shorter duration. The rapid-acting component of Humalog Mix50/50, is absorbed faster than Regular human insulin (U-100).Insulin lispro protamine (or) insulin lispro is used along with a proper diet and exerciseprogram to control high blood sugar. It is used in people with type 1 (insulin-dependent) or type 2 diabetes. This product is a combination of two man-made insulins: intermediate-acting insulin lispro protamine and rapid-acting insulin lispro. This combination starts working faster and lasts for a longer time than regular insulin.

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