

Due to complexity of protein drugs, no single analytical method can detect all possible chemical, physical, and immunological changes in the protein structure. Thus, several analytical techniques such as electrophoresis, spectroscopy, chromatography, thermal analysis, immunoassays, and bioassays may be required to completely characterize a protein and its degradation profile. While recombinant DNA technology and other biotechnology principles have revolutionized the production of proteins, their analysis is still largely dependent on traditional assay methodology.

General methods for the estimation of protein drugs are reviewed below:

ELECTROPHORESIS

Commonly used electrophoretic analytical techniques for the analysis of peptides and proteins include SDS-PAGE, isoelectric focusing, and capillary electrophoresis. Proteins because of their charge migrate under the influence of an applied electric field. The movement of protein under electrophoresis depends on their mass-to-charge ratio. Following electrophoresis, the protein can be visualized and quantitated by coomassie blue or silver staining or Western blot or enzymatically labeled or radiolabeled antibody.

SPECTROPHOTOMETRIC DETERNINATION

The absorption spectrum of a protein in the UV range (175-350 nm) is a net result of absorption of light by the carboxyl group of peptide bond (190-210 nm), the aromatic amino acids (250-320 nm), and the disulfide bonds (250-300 nm). Secondary, tertiary and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength etc. can alter the absorbance spectrum. UV spectroscopy can be used to measure protein content without the need for any calibration with standards. Any protein with at least one tryptophan residue can be detected by UV spectroscopy at concentration of 0.1 mg/ml or more. The disadvantage with this method is that other compounds may absorb these wavelengths e.g. a number of compounds such as carboxylic acids, buffer ions, alcohols, bicarbonate and aromatic compounds absorb at UV light at max of around 200 nm. Also nucleic acids absorb in the range of about 280-nm

FLUORESCENCE SPECTROSCOPY

The aromatic amino acids, Phe, Try, and Tyr exhibit fluorescence. The fluorescence is in the order Trp > Tyr > Phe. Fluorescence of Tyr is generally observed only in the absence of Trp, while that of Phe is observed only in the absence of both Tyr and Trp. Most proteins exhibit fluorescence in the 300 - 400 nm range when excited at 250 - 300 nm. Fluorescence is much more sensitive to protein confirmation as compared to UV spectroscopy. The indole side chain of Try is the largest and most fluorescent of all amino acids. Also most proteins have only one or just a few residues of Trp, and these hydrophobic residues are generally buried in the interior of the folded protein. Therefore, unfolding of a protein molecule is often accompanied by an increase in the fluorescence can also be used as a probe of protein-peptide interactions.

COLORIMETRIC METHODS

Absorbance of light in the visible range is due to conjugated double bond systems and forms the basis for colorimetric assays. Although peptides and proteins do not absorb visible light, they can react with reagents to form colored compounds.

a) LOWRY METHOD

This is the most widely used method for the quantitative determination of proteins. The method of Lowry et al is based on the folin phenol reagent of folin and Ciocalters, the active constituent of which is phosphomolybdic-tungstic mixed acid. The mixed acids in the folin-Ciocalters preparation are the final chromagen and involve the following chemical species.

3 H₂O X P₂O₅ X 13 WO₃ X 5 MoO₃ X 10 H₂O

3 H₂O X P₂O₅ X 14 WO₃ X 4 MoO₃ X 10 H₂O

Proteins effect a reduction of the mixed acid by a loss of 1, 2 or 3 oxygen atoms from tungstate and /or molybdate, thereby producing one or more of senerable possible reduced species, which have a characteristic blue color (λ max 745 -750nm). Copper apparently chelates in the peptide structure and facilitates electron transfer to the mixed acid chromagen, particularly in the vicinity of amino acid functional groups, thereby increasing the sensitivity to protein.

However, the principal disadvantage of this method is lack of specificity. Many substances are known to interface with this method. The other disadvantages include slow reaction rates, reagent instability and nonlinearity [1,2].

b) BICINCHONINIC (BCA) METHOD

The use of bicinchoninic acid to determine protein concentration is rapidly gaining favor as an alternative to the Lowry method of protein determination. The sensitivity of the technique and its case of use make the BCA assay preferable to the Lowry assay in many instances. The BCA assay has also been reported to be less sensitive to interference by a number of commonly encountered compounds [3].

The assay utilizes the color change resulting from the strong complex formed between Cu^+ and BCA. Smith and coworkers have postulated that Cu^+ is produced by two separate reactions. The first is temperature independent and is postulated to arise from the oxidation of cysteine, tyrosine and tryptophan residues. The second source of Cu is assumed to be a temperature dependent reaction of peptide bonds with Cu⁺. This process is reported to be more prevalent as the incubation temperature is increased [3].

Electrochemical studies and the magnitude of the color changes observed when the reaction is carried out at 37°C indicate that tryptophan, tyrosine, and the peptide bond are not completely oxidized at this temperature. When the reaction

temperature is increased to 60°C, significantly more color formation is observed for these three groups.

Stoscheck has suggested that the BCA assay will replace the Lowry because it requires a single step, and the color reagent is stable under alkaline condition [4].

Range : > 9.2 micrograms

Volume : 1 ml (scale up for larger cuvettes)

Accuracy : Good

Convenience : Good

Major interfering agents: Strong acids, ammonium sulfate, and lipids.

c) BRADFORD ASSAY

The Bradford assay is a rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein dye binding.

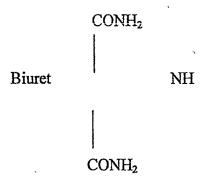
The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retest within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentration for gel electrophoresis [5].

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye albumin complex solution is constant over a 10-fold concentration range [5].

d) BIURET METHOD

Alkaline copper sulfate reacts with compounds containing two or more peptide bonds to give a violet-colored complex. The depth of the color obtained is a measure of the number of peptide bonds present in the protein. The name of the test comes from the compound biuret, which gives a typical positive reaction.

The reaction is not absolutely specific for peptide bonds, since any compound containing two carbonyl groups linked through nitrogen or carbon atom will give a positive result.



e) NINHYDRIN REACTION

Ninhydrin, a powerful oxidizing agent, reacts with all amino acids between pH 4 and 8 to give a purple colored compound. The reaction is very sensitive and is ideal for the detection of amino acids.

SEMI MICRO KJELDAHL METHOD FOR DETERMINATION

This method based on determination of Nitrogen. It involves the digestion of aliquot of sample solution along with conc. H_2SO_4 and 30% hydrogen peroxide solution, till no carbonaceous material remains. After digestion is complete distillation is done and finally titration is carried out with sulfuric acid to get a slight purple color. This method is modification of the USP NFXX nitrogen determination by method II.

IMMUNOASSAY

Radioimmunoassay (RIA) exploits the specific and tight association of antibody with a peptide or protein drug as the antigen, to determine very low concentrations of the drug. In carrying out the assay, the unlabeled protein is exposed to its antibody in the presence of a known amount of radiolabeled protein. The concentration of protein is determined by measuring the unbound hot ligand after separating from the bound ligand. RIA has been used as an analytical probe to assay and to detect structural changes in cholecystokinin, insulin [6], somatostatin, and vasopressin. Another popular immunoassay is the enzyme-linked immunosorbent assay (ELISA). A commonly used form of ELISA is the double antibody sandwich assay that utilizes two antibodies. The second antibody has an enzyme such as horseradish peroxidase or alkaline phosphatase, which, with an develop color and can be appropriate substrate, can analyzed spectrophotometrically. Thus, ELISA does not need a radiolabeled protein for the assay. Since the interaction between protein and antibody involves specific sites, immunoassays are sensitive to conformational changes in the protein. These assays indicate immunoactive site may not affect the bioactivity of the protein, but it will lead to a drop in its assay value by immunoassay. Similarly, a change in structure or conformation that reduces the bioactivity of the protein may not affect its value by RIA, as long as the antigenic site is intact and capable of interacting with the antibody. As an example, the sites on the insulin molecule that act as immunogenic determinants and sites that bond to biological receptors are different. This led to the development of a radioreceptor assay for insulin, which uses the biological receptor for insulin, as a basis for assay.

ELISA assays were found to be more sensitive and reproducible. But are less predictive of pharmacodynamics of the drug, compared to bioassays.

RADIO RECEPTOR ASSAY

The source of the receptor chosen for the radio receptor assay was the cultured human lymphocyte cell line first suggested for assay used by Gavin and coworkers [1].

In the assay, insulin binding is detected by using a competition between unknown or reference insulin and insulin labeled with ^{125}I . The amount of radioactivity bound to the cells is the measured parameter as in RIA [7].

The higher the insulin concentrates in the test material, the lower the binding of 125 I.

In addition to the methods mentioned under quantitative determination of peptides & proteins, following methods can be employed for the determination of insulin.

BIOASSAYS

The potency of protein drugs are estimated by comparing in given conditions their biological activity it produces with that produced by the International Reference preparation of the drug or a reference preparation calibrated in International Units. The biological effect exerted directly related to their concentration in the blood. Various kits are available in the market, for measuring their effect in the biological fluids. Also, specific methods are available to measure the biological constituents of the blood, which give the amount of the drug. These methods are more specific, more easy and accurate.

For example, by measuring the hypocalcemic effect in the blood using atomic absorption spectroscopy, calcitonin can be estimated.

Atomic absorption spectrometry is a method for determining the concentration of an element in a substance by measuring the absorption of radiation by atomic vapour of the element generated from the substance. The determination is carried out at the wavelength of one of the absorption lines of the element concerned.

CHROMATOGRAPHY

In high-pressure liquid chromatography, the interactions between the column packing and the hydrophobic regions of the protein are exploited. A majority of separations are performed on large pore size, silica-based stationary phases containing chemically bonded alkyl chains of various length, typically C_4 , C_8 , or C_{18} . Other chromatographic techniques include size exclusion chromatography, ion

exchange chromatography, hydrophobic interaction chromatography, and affinity chromatography.

In this research work, following methods were employed for the determination of insulin and calcitonin.

3.1 ESTIMATION OF INSULIN IN BLOOD

The potency of insulin is estimated by comparing in given conditions the hypoglycemic effect it produces with that produced by the known quantity of drug in animals. The bioactivity of the insulin i.e. the reduction in blood glucose levels as a function of measuring the drug concentration. The blood glucose level is measured, which is proportional to the insulin concentration in the blood.

PRINCIPLE OF GOD/POD METHOD

The aldehyde group of glucose is oxidized by Glucose oxidase (GOD) to gluconic acid and hydrogen peroxide in the presence of peroxidase (POD) is broken down to water & oxygen. The oxygen reacts with 4-amino phenazone in the presence of phenol to from a red colored quinoneamine compound. The intensity of the color is proportional to the concentration of glucose in the solution and can be measured spectrophotometrically (470 to 540nm) [8].

GOD D-glucose + O_2 \longrightarrow Gluconic acid + H_2O_2 H_2O POD H_2O_2 + PAP \longrightarrow Red dye + H_2O

The method is enzymatic, so it is very specific and sensitive.

ESTIMATION OF GLUCOSE BY GOD/POD METHOD

Equipments

The equipments used for the estimation of Glucose include Clinical Chemistry Analyzer RA-50 (Miles Inc. Diagnostic Division, Elkhart, In, USA), Calibrated pipettes 0.1ml, 0.2ml, 0.5ml, 1.0ml, 5.0ml, 10ml; Volumetric flasks of 10ml, 50ml and 100ml. capacity and other glassware of borosil.

Materials

Glucose Estimation Kit (GOD/POD method) (Bayer's Diagnostics India Ltd, India), Purified water and control serum.

Reagents

Glucose Working Reagent:

It is prepared by mixing 100 parts of buffer solution present in the Kit and 1 part of enzyme concentrate of the kit.

Standard Solution of Glucose

It is provided in the kit (100mg/dL).

Procedure

The Standard Solution of Glucose is used for calibrating the Clinical Chemistry Analyzer. 10μ l of Standard solution of Glucose is taken and to it 500µl of Glucose working Reagent is added. It is allowed to stand for 10 minutes and then analyzed. Once equipment is calibrated, samples can be read for glucose by employing the same procedure.

General System Parameters

Reaction Type : Endpoint

Reaction Slope	: Increasing
Wavelength	: 505 nm
Flowcell Tem.	: 30°C
Incubation	: 15 min. at 37°C or 30 min at RT
Sample Vol.	: 10µl
Reagent Vol.	:1.0mL
Std. Concentration	: 100 mg/dL
Zero setting with	: Reagent Blank

QUALITY CONTROL

Commercial reference control serum that contains glucose was diluted with glass double distilled water to the dilution of 10 mg/dL, 20 mg/dL, 40 mg/dL, 60 mg/dL, 80 mg/dL, 100 mg/dL, 120 mg/dL, 200 mg/dL, 300 mg/dL, 500 mg/dL

 10μ l of prepared reference control serum is taken and to it 500μ l of Glucose working Reagent is added. It is allowed to stand for 15 minutes and then analyzed. Once equipment is calibrated, samples can be directly read for glucose content present in mg/dL.

3.2 ESTIMATION OF INSULIN IN DIFFUSION STUDIES BY BCA METHOD

EQUIPMENTS

pH meter (Global Electronics, Hyderabad, India), Analytical balance (Precisa 205 ASCS, Switzerland), Calibrated pipettes, Clinical chemistry analyzer RA-50 (Miles Inc. Diagnostic Division, Elkhart, In, USA). Volumetric flasks of 10 ml, 50 ml capacity and other glassware of borosil.

MATERIALS

Insulin Porcine (25.5 IU/mg) (Sarabhai Chemicals, Vadodara), Citric acid anhydrous (extra pure), Sodium tauroglycocholate, Sorbiton tri-oleate, Sodium caprylate (S.D. fine-chem. Ltd. India), Oleic acid (cis 9-ocatadecanoic acid) sodium salt, Bacitracin, Bestatin, and Chymostatin (Sigma-Aldrich Chemie GmbH, Germany). All chemicals were used without further testing.

REAGENTS

- 1. Protein estimation Kit (BCA) (Genei Pvt.Ltd, Bangalore, India),
- 2. Phosphate buffer pH 7.4, Phosphate buffer pH 6.0 and Citrate buffer pH 3.5.
- 3. Frog ringer's solution.

BCA WORKING REAGENT

This was prepared by mixing Reagent A (50 parts) with Reagent B (1 part) of the protein estimation Kit by BCA method.

PREPARATION OF INSULIN SOLUTION IN PHOSPHATE BUFFER pH 7.4

5.0 mg of porcine insulin was accurately weighed and dissolved in 50 ml of phosphate buffer pH 7.4. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively (Table 3.2).

PREPARATION OF INSULIN SOLUTION IN PHOSPHATE BUFFER pH 6.0

5.0 mg of porcine insulin was accurately weighed and dissolved in 50 ml of phosphate buffer pH 6.0. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively (Table 3.2).

PREPARATION OF INSULIN SOLUTION IN CITRATE BUFFER

5.0 mg of porcine insulin was accurately weighed and dissolved in 50 ml of citrate buffer pH 3.5. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively (Table 3.2).

PREPARATION OF INSULIN SOLUTION IN FROG RINGER'S SOLUTION

5.0 mg of porcine insulin was accurately weighed and dissolved in 50 ml frog ringer's solution. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively (Table 3.2).

STANDARD SOLUTION OF PROTEIN

It is provided in the kit.

PROCEDURE

Pipette 0.5 ml of each samples into appropriately labeled test tubes. 0.5 ml of the buffer (diluent) was used for blank. 0.5 ml of the BCA working reagent was added to each vial & mixed well. All the tubes were incubated at 60 °C for 30 minutes.

After incubation, all tubes were cooled to room temperature. The samples were aspired through the aspiration tube in the chemistry analyzer at a wavelength of 561.5 nm. The calibration of the instrument done by passing the standard solution provided in the assay kit. The analysis was done in triplicate and mean S.D. of the values was calculated.

CHECKING FOR INTERFERENCE

Insulin solutions containing penetration enhancers (concentrations maximum of used in the formulations) i.e. Sodium tauroglycocholate, Sorbiton tri-oleate, Sodium caprylate, Oleic acid (cis 9-ocatadecanoic acid) sodium salt, Bacitracin, Bestatin, and Chymostatin were prepared in 10 ml volumetric flasks. Then the procedure as mentioned in the above paragraph was followed.

3.3 ESTIMATION OF INSULIN IN FORMULATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Mobile Phase A

Mobile phase A was prepared by dissolving 28.4 g of anhydrous sodium sulphate R in water R and diluted to 1000 ml with the same solvent; 2.7 ml of phosphoric acid R was added; pH adjusted to 2.3, if necessary with ethanolamine R; filtered and degassed;

Mobile phase B

550 ml of mobile phase A mixed with 450 ml of acetonitrile R. The solution was warmed to a temperature of at least 20°C in order to avoid precipitation (mixing mobile phase A with acetonitrile is endothermic). The solution was filtered and degassed.

Mobile phase

Mobile phase was prepared by mixing 42 volumes of mobile phase A and 58 volumes of mobile phase B.

Column

- size: l = 0.25, $\emptyset = 4.6$ mm
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μm),
- temperature: 40°C

Flow rate: 1 ml/min.

Detection: Spectrophotometer at 214 nm.

Standard Preparation

Dissolve an accurately weighed quantity of the appropriate reference standard insulin (pork) RS in 0.01 N hydrochloric acid to obtain a solution having a known concentration corresponding to that of assay preparation.

Procedure

Separately inject equal volumes (about 20 μ l) of the standard preparation and the assay preparation into the chromatograph, record the chromatograms, and measure the responses for the major peaks (Table 3.3).

3.4 ESTIMATION OF CALCITONIN IN BLOOD

The potency of calcitonin is estimated by comparing in given conditions the hypocalcaemic effect it produces with that produced by the International reference preparation of salmon calcitonin or a reference preparation calibrated in International Units. The International Unit is the activity contained in a stated amount of the International reference preparation, which consists of a quantity of freeze-dried synthetic salmon calcitonin with mannitol. The equivalence in International Units of the International Standard is stated by the World Health Organization (WHO).

The reduction in blood calcium level can be measured by Cresolphthalein Complexone Method.

Cresolphthalein Complexone Method

Calcium, in an alkaline medium, reacts with o-cresolphthalein complexone to form an intense chromophore that absorbs light at 575nm (570-580nm). Magnesium and iron are excluded from the reaction by complexing with 8-Hydroxyquinoline [9,10].

Equipment

The major equipments used for the estimation of calcium include Clinical Chemistry Analyzer RA-50 (Miles Inc. Diagnostic Division, Elkhart, IN, USA), Calibrated pipettes 0.1ml, 0.2ml, 0.5ml, 1.0ml, 5ml, 10ml; Volumetric flasks of 10 ml., 50 ml capacity and other glass ware of Borosil.

Materials

Calcium Estimation Kit (o-cresolphthalein complexone) (Bayer Diagnostics India Ltd., Baroda, India), Purified water

Standard Solution of Calcium

It is provided in the kit (10mg. /dl.).

Procedure

The Standard solution of calcium is used for calibrating the Clinical chemistry Analyzer. 10μ l of standard solution of calcium is taken and to it 250 μ l of O-Cresolphthalein complexone reagent (Reagent 1) and 250 μ l. of buffer solutions (Reagent 2) are added. It is allowed to stand for 15 minutes and then analyzed. Once equipment is calibrated, samples can be read for calcium by employing the same procedure.

General System Parameters

Reaction Type	: Endpoint
Reaction Slope	: Increasing
Wavelength	: 575 nm
Flowcell Tem.	: 30°C

Incubation	: 5 min. RT
Sample Vol.	: 10µ1
Reagent Vol.	: 1.0mL
Std. Concentration	: 10 mg/dL
Zero setting with	: Reagent Blank

QUALITY CONTROL

Commercial reference control serum of calcium diluted with glass double distilled water to the dilution of 2.0 mg/dL, 4.0 mg/dL, 6.0 mg/dL, 8.0 mg/dL, 10.0 mg/dL

10 μ l of prepared reference control serum is taken and to it 250- μ l.of o-Cresolphthalein complexone reagent (Reagent 1) and 250 μ l. of buffer solutions (Reagent 2) are added. It is allowed to stand for 15 minutes and then analyzed. Once equipment is calibrated, samples can be read for calcium.

3.5 ESTIMATION OF CALCITONIN IN DIFFUSION STUDIES BY BCA METHOD

Equipments

The major equipments used for the estimation calcitonin include pH meter (Global Electronics, Hyderabad, India), Analytical balance (Precisa 205 ASCS, Switzerland), Micropippettes, Clinical Chemistry Analyzer RA-50 (Miles Inc. Diagnostic Division, Elkhart, In. USA). Volumetric flasks of 10 ml 50 ml capacity and other glassware of Borosil.

Materials

Salmon calcitonin (6123 IU/mg) was kindly gifted by Novartis Pharma AG, Basel (Switzerland). Dimethyl β -cyclodextrin, dodecyl maltoside, oleic acid (cis 9-ocatadecanoic acid) sodium salt, bestatin, amastatin, chymostatin, bacitracin were

purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany); sodium tauroglycocholate was purchased from S.D. fine-chem. Ltd (Mumbai, India).

All chemicals were used without further testing.

Reagents

BCA - kit (Genei Pvt.Ltd, Bangalore, India), Acetate buffer pH 6.0; acetate buffer pH 3.9 and acetate buffer pH 2.8, Frog ringer's solution.

BCA Working Reagent

This was prepared by mixing Reagent A (50 parts) with Reagent B (1 part) of the protein estimation Kit by BCA method.

Preparation of calcitonin solution in Acetate buffer pH 6.0

5.0 mg of calcitonin was accurately weighed and dissolved in 50 ml of phosphate buffer pH 7.4. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively.

Preparation of calcitonin solution in acetate buffer pH 3.9

5.0 mg of calcitonin was accurately weighed and dissolved in 50 ml of citrate buffer pH 3.5. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively.

Preparation of calcitonin solution in acetate buffer pH 2.8

5.0 mg of calcitonin was accurately weighed and dissolved in 50 ml of citrate buffer pH 3.5. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken

and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively.

Preparation of calcitonin solution in frog ringer's solution

5.0 mg of călcitonin was accurately weighed and dissolved in 50 ml frog ringer's solution. From this 0.1 ml, 0.5 ml, 1.0 ml, 2.0 ml, 3.0 ml, 5.0 ml, was taken and transferred into separate 10 ml volumetric flasks. The volume was made up with the buffer. The resulted solution contains 1.0 μ g/ml, 5.0 μ g/ml, 10 μ g/ml, 20 μ g/ml, 30 μ g/ml, and 50 μ g/ml respectively.

Standard Solution of Protein

It is provided in the kit.

Procedure

Pipette 0.5 ml of each sample into appropriately labeled test tubes. 0.5 ml of the buffer (diluent) was used for blank. 0.5 ml of the BCA working reagent was added to each vial & mixed well. All the tubes were incubated at 60 °C for 30 minutes.

After incubation, all tubes were cooled to room temperature. The samples were aspired through the aspiration tube in the chemistry analyzer at a wavelength of 561.5 nm. The calibration of the instrument done by passing the standard solution provided in the kit and confirmed that the analyzer shows the protein content of the standard. The analysis was done in triplicate and mean S.D. of the values was calculated.

Checking for Interference

Calcitonin solutions containing highest concentrations of the penetration enhancers i.e. Sodium tauroglycocholate, Sorbiton tri-oleate, Sodium caprylate, Oleic acid sodium salt, Bacitracin, Bestatin, and Chymostatin used in the formulations were prepared in 10ml volumetric flasks. Then the procedure mentioned above in the paragraph was followed.

3.6 ESTIMATION OF CALCITONIN IN FORMULATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Mobile phase A

3.26 g of tetramethylammonium hydroxide R in 900 ml of water R, pH adjusted to 2.5 with phosphoric acid R and mix with 100 ml of acetonitrile for chromatography R; filter.

Mobile phase B

Dissolve 1.45 g of tetramethylammonium hydroxide R in 400 ml of water R, adjust the pH to 2.5 with phosphoric acid R and mix with 600 ml of acetonitrile for chromatography R; filter and degas.

Standard Preparation

Dissolve an accurately weighed quantity of the appropriate Reference Standard of calcitonin (salmon) RS in the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 μm),
- a mobile phase at a flow rate of 1.0 ml/min.
- as detector a spectrophotometer set at 220 nm,
- temperature of the column at 65°C
- Equilibrate the column with a mixture of 72 volumes of mobile phase A and 28 volumes of mobile phase B.

Time (min)	Mobile phase A	Mobile phase B	Comment
	(percent V/V)	(percent V/V)	
0-30	72 → 48	28 → 52	Linear gradient
30-32	48 → 72	$52 \rightarrow 28$	Switch to initial eluent composition
32-55	72	28	Re-equilibration

Procedure

Separately inject equal volumes (about 20 μ l) of the standard preparation and the assay preparation into the column, record the chromatograms, and measure the responses for the major peaks.

3.7 RESULTS AND DISCUSSION:

All the analysis was done in triplicate. In Table 3.1 the glucose content of the samples of reference control serum were recorded. Each of the sample concentration was analyzed three times (sample 1, sample 2 and sample 3 in Table 3.1) and the mean of three values along with their standard deviations was recorded. The amount of glucose was directly read from the analyzer in mg/dL. The variation in the glucose concentration of the analyzed samples was $\pm 2.0\%$ of the original concentration of the prepared solution. The method is linear up to 500 mg/dL. The use of quality control material checks both, the instrument and the reagent functions together.

In Table 3.2 the insulin solution prepared in different buffer that are used in the formulations were shown. The amount of insulin was directly read from the analyzer in μ g/ml. The obtained mean values of insulin content of the standard samples of conc. from 1.0 - 50.0 μ g/ml were recorded. Each of the sample concentration was analyzed three times (sample 1, sample 2 and sample 3 in Table 3.2) and the mean of three values along with their standard deviations was

recorded. The variation in the insulin concentration of the analyzed samples was \pm 5.0% of the original concentration of the prepared solution. The method was linear up to 50.0 mg/dL. The interference of the excipients used was found to be below 1.0 %.

The amount of insulin in the formulations calculated from the HPLC chromatogram obtained for reference and the sample.

Sample Peak Area

The amount of insulin = _____ X Standard weight Standard Peak Area

The linearity of the method was checked by injecting insulin solution at four concentrations, 0.1 μ g, 1.0 μ g, 10 μ g and 100 μ g. Each of the sample concentration was injected three times (sample 1, sample 2 and sample 3 in Table 3.3) and the mean of three values along with their standard deviations was recorded. The concentration of the injected solution was plotted against mean peak area in Figure 3.1. The r² value of solutions of all the concentration levels were found to be 1.0 indicate the linearity of the method.

In Table 3.4 the calcium content of the samples of reference control serum were recorded. Each of the sample concentration was analyzed three times and the mean of three values along with their standard deviations is recorded. The amount of calcium is directly read from the analyzer in mg/dL. The variation in the calcium concentration of the analyzed samples was $\pm 2.0\%$ of the original concentration of the prepared solution. The method is linear up to 15 mg/dL. The use of quality control material checks both, the instrument and the reagent functions together.

In Table 3.5 the calcitonin prepared in different buffer that are used in the formulations were shown. The amount of calcitonin directly read from the analyzer in μ g/ml. The obtained mean values of calcitonin content of the samples of conc. from $1.0 - 50.0 \mu$ g/ml were recorded. Each of the sample concentration was analyzed three times and the mean of three values along with their standard deviations is recorded. The variation in the calcitonin concentration of the analyzed samples was \pm 5.0% of the original concentration of the prepared solution. It is

up to 50.0 mg/dL. The interference of the excipients used was found to be below 1.0 %.

The amount of calcitonin in the formulation calculated from the HPLC Chromatogram obtained for reference and the sample.

Sample Peak Area

The amount of calcitonin = _____ X Standard weight

Standard Peak Area

The content of calcitonin (salmon) was calculated from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of $C_{145}H_{240}N_{44}O_{48}S_2$ in calcitonin CRS. Proceed with tangential integration of the peak areas. The linearity of the method was confirmed by injecting calcitonin at four concentrations, 0.1 µg, 1.0 µg, 10 µg and 100 µg. Each of the sample concentration was injected three times (sample 1, sample 2 and sample 3 in Table 3.6) and the mean of three values along with their standard deviations was recorded. The concentration of the injected solution was plotted against mean peak area in Figure 3.2. The r^2 value of solutions of all the concentration levels were found to be 1.0 indicate the linearity of the method.

S.No.	Conc. of glucose in	Amount	Mean value		
	reference control		(mg/dL)		of glucose in
	serum (mg/dL)	Sample 1	Sample 2	Sample 3	mg/dL
1	10	10.1	10.1	10.0	10.07 ±0.06
2	20	19.8	19.9	20.1	19.93±0.15
3	40	40.0	40.4	40.3	40.23±0.21
4	60	59.1	60.8	61.0	60.30±1.04
5	80	81.3	81.6	80.4	81.1±0.62
6	100	98.2	99.5	9 9.7	99.13±0.81
7	120	120.9	121.4	122.1	121.47±0.60
8	200	198.3	199.5	200.4	199.40±1.05
9	300	302.9	300.0	298.1	300.33±2.42
10	500	501.5	499.0	498.7	499.73±1.54

ESTIMATION OF BLOOD GLUCOSE LEVEL IN SERUM BY GOD/POD METHOD

TABLE 3.1

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Solution for	Conc. in	Amoun	Mean value		
analysis	µg/ml		(µg/ml)		
		Sample 1	Sample 2	Sample 3	µg/ml
	. 1	1.05	1.03	0.98	1.02±0.04
	5	5.02	4.96	5.14	5.04±0.09
Insulin in phosphate	10	9.65	9.99	10.0	9.88±0.20
buffer pH 7.4	20	20.07	20.0	20.11	20.06±0.06
	30	30.12	30.0	30.03	30.05±0.06
	50	49.2	51.0	50.08	50.90±0.05
	1	0.96	1.01	1.05	1.05±0.05
	5	5.07	5.02	4.89	4.99±0.09
Insulin in phosphate	10	10.24	9.92	10.00	10.05±0.17
buffer pH 6.0	20	19.89	19.92	19.71	19.84±0.11
	30	30.15	30.09	30.14	30.12±0.03
	50	51.0	50.91	50.10	50.67±0.50
	1	1.06	1.02	0.97	1.02±0.05
	5	4.95	5.00	4.94	4.96±0.03
Insulin in citrate	10	10.32	10.00	10.21	10.18±0.16
buffer pH 3.5	20	19.1	20.20	20.10	19.8±0.61
	30	30.3	30.10	29.45	29.95±0.44
	50	49.2	49.92	50.05	49.72±0.46
	1	1.01	1.05	0.94	1.0±0.06
Insulin in Frog	5	5.0	5.02	4.90	4.97±0.06
ringer's solution	10	10.2	10.03	9.89	10.04±0.16
	20	20.2	19.28	19.91	19.80±0.48
	30	30.0	30.8	29.88	30.23±0.50
	50	50.4	50.9	50.22	50.52±0.35

ESTIMATION OF INSULIN BY BCA METHOD

TABLE 3.2

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S.No	Conc. of	Peak Area	of the prepa	Mean Peak	
	insulin injected		solution	Area	
	(µg/ml)	Sample 1	Sample 2	Sample 3	
1	0.10	9580	9639	9612	9610 ± 30
2	1.00	96524	96572	96402	96499 ± 88
3	10.0	967154	967859	967352	967455 ± 363 ,
4	100.0	9654270	9654952	9654412	9654544 ± 360
	r ²	1.0	1.0	1.0	1.0

ESTIMATION OF PEAK AREA OF ISULIN BY HPLC METHOD

TABLE 3.3

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ESTIMATION OF BLOOD CALCIUM LEVEL IN SERUM BY ORTHO-CRESOLPHTHALEIN COMPLEXONE METHOD

S.No.	Conc. of calcium in	Amount of c	Amount of calcium detected (mg/dL)			
	reference control	Sample 1	Sample 2	Sample 3	of calcium	
	serum (mg/dL)			-	(mg/dL)	
1	2	2.02	1.98	2.05	2.02±0.04	
2	4	4.08	4.00	4.11	4.06±0.07	
3	6	5.95	5.91	6.08	5.98±0.09	
4	8	8.02	8.07	7.99	8.03±0.04	
5	10	10.03	10.15	9.99	10.06±0.08	
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TABLE 3.4

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Standard solution	Conc. in	Amount	Amount of calcitonin detected				
for analysis	µg/ml		(µg/mi)				
		Sample 1	Sample 2	Sample 3	(µg/ml)		
	1	1.03	0.97	1.07	1.02±0.05		
	5	5.00	5.12	4.97	5.03±0.08		
Insulin in phosphate	10	10.11	10.0	9.98	10.03±0.07		
buffer pH 7.4	20	19.85	20.09	20.03	19.99±0.12		
	30	30.32	29.86	30.02	30.06±0.23		
	50	50.02	49.01	49.12	49.38±0.55		
	1	1.02	1.04	0.98	1.01±0.03		
	5	5.00	5.00	4.87	4.96±0.08		
Insulin in phosphate	10	10.16	10.12	10.02	10.10±0.07		
buffer pH 6.0	20	19.99	19.97	20.21	20.06±0.13		
	30	30.25	30.21	30.00	30.15±0.13		
	50	50.11	50.02	49.65	49.93±0.24		
	1	1.12	1.02	0.96	1.02±0.05		
	5	5.24	5.17	4.91	4.96±0.03		
Insulin in citrate	10	10.44	10.22	9.61	10.18±0.16		
buffer pH 3.5	20	19.21	20.31	20.19	19.8±0.61		
	30	30.51	30.00	29.15	29.95±0.44		
	50	49.85	49.77	50.12	49.72±0.46		
	1	1.05	1.00	0.96	1.00±0.05		
Insulin in Frog	5	5.20	5.12	4.97	5.10±0.12		
ringer's solution	10	9.75	10.07	10.21	10.01±0.24		
	20	20.12	19.35	19.91	19.79±0.40		
	30	29.00	30.0	29.78	29.59±0.53		
	50	50.49	50.94	50.72	50.72±0.23		

ESTIMATION OF CALCITONIN BY BCA METHOD

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TABLE 3.5

S.No	Conc. of	Peak A	Mean Peak		
•	Calcitonin	Cal	Area		
	injected (µg/ml)	Sample 1 Sample 2 Sample 3			
1	0.10	6090	6140	6099	6110 ± 27
2	1.00	60752	60705	60710	60722 ± 26
3	10.00	607350	607466	607448	607421 ± 62
4	100.00	6072248	6072512	6072363	6072374 ± 132
	r ²	1.0	1.0	1.0	1.0

ESTIMATION OF PEAK AREA OF CALCITONIN BY HPLC METHOD

TABLE 3.6

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STANDARD CURVE OF INSULIN BY HPLC METHOD

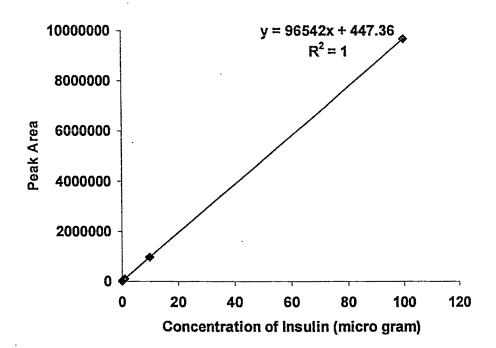


FIGURE 3.1

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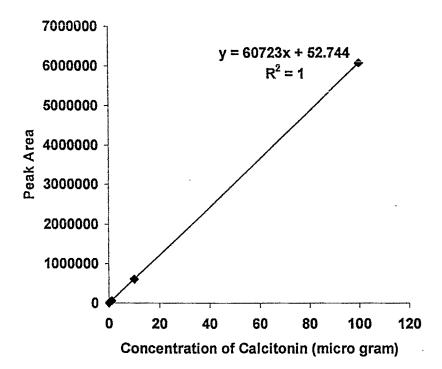


FIGURE 3.2

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