Review Article

International Journal of Research in Pharmaceutical Sciences

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Review on Analytical Methods for the Determination of Insulin

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Article History:

Received on: 05 Apr 2020
Revised on: 07 Apr 2020
Accepted on: 09 Apr 2020

Keywords:

Insulin,
Immunoassays,
ELISA and CLIA

Abstract

Insulin is a major hormone of polypeptides that regulates the metabolism of carbohydrates. Diabetes mellitus, characterized by chronic hyperglycemia, is associated with excessive levels of insulin. As a result, accurate insulin quantification is important for hyperglycemia diagnosis and care. Insulin's main purpose is to keep blood sugar levels in check. Insulin performs its functions by binding to nerve terminals on liver,fat, and striated muscle. In response to an increase in blood glucose levels, insulin facilitates the migration of the endothelial glycosyltransferases type iii (GLUT4) to a cellular membranes, increasing glucose absorption in insulin-sensitive periphery tissues such as fat and muscle. The activation of liver glycogenesis is then aided by insulin.Finally, insulin signals the liver to avoid processing glucose by preventing both glycogenolysis and gluconeogenesis. Where there is a low level of glucose in the blood, glycogens are converted to glucose and released into the bloodstream. The key instruments for detecting insulin have been immunochromatographic techniques. The current research focuses on the different analytical methods for assessing Insulin levels. This study looked at assay methods (AlphaLISA,ELISA,RIA,CLIA, HTRF, and on-chip motilal oswal financial), column chromatography immunoassay (LC-MS/MS andHPLC-UV, MECC,), but also novel science, industrial, and research biosensor assays. ELISA has long been a standard instrument in therapeutic immunoassays because of its high output. On the other hand, advanced computational methods, including such spectroscopy techniques, are incredibly specific but promising instruments that can differentiate between glucose and its analogues. The advantages and disadvantages of the most common methods were also discussed.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v12i2.4710

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INTRODUCTION

Protein is a cysteine hormone that is made up of a disulfide-bonded string of 21 acids A and a string of 30 proteins B. Insulin is a well-known biopharmaceutical that is used to treat both type 1 and type 2 diabetes. The two insulin links create a large packed arrangement with tubular sections in each of them, and so do the separate glucose chains.Insulin is a key active metabolite that is controlled by the pancreas' beta cells and promotes the body's carbon-based energy storage (Ogbera, 2014).

The precursor to insulin, proinsulin, has 86 residues

and in type of a chain, an B chain, or a C-Peptide connector. Cleavage of C peptide proinsulin initiates insulin biosynthesis, which results in the development of insulin (Figure 1). Insulin's main purpose is to keep blood sugar levels in check. Insulin performs its functions by binding to various receptors on fat, liver, and muscle cells. In response to an increase in blood sugar levels, insulin facilitates the migration of the endothelial glycosyltransferases type 4 (GLUT4) to a cellular membranes, increasing glucose absorption to oestrogen secondary tissue and muscle and fat.Insulin then assists in the activation of liver glycogenesis. Finally, insulin signals the liver to avoid processing glucose by preventing both glycogenolysis and gluconeogenesis. Where there is a low level of glucose in the blood, glycogens are converted to glucose and released into the bloodstream (Arnolds *et al.*, 2010).

Diabetes mellitus (DM) is a metabolic condition characterised by recurrent hyperglycemia caused by insulin resistance or release. Form I and type II diabetes are the two forms of diabetes. The pancreatic defence mechanism of the -cell body is degraded in type I diabetes. As a result, little or no insulin is released, causing problems with glucose metabolism. Diabetes type II is caused by insulin resistance, or a lack of sufficient blood glucose control. Exogenous insulin has long been used to regulate blood glucose levels and alleviate diabetic symptoms. While human beings can be synthesised, their physiology is constrained by their propensity to interact with condensed solutions for the production of dimers and hexamers. Researchers created rDNA human insulin analogues with a lower proclivity to self-associate and improved treatment efficacy in order to improve insulin production (Baynes, 2015). Insulin analogues are human insulin molecules that have been modified. Fast-acting insulin analogues (insulin lispro, insulin aspart, and insulin glulisine) and long-acting insulin analogues (insulin glulisine) are the two main forms of insulin analogues (insulin glargine and insulin detemir) (Figure 1) (Yehezkel *et al.*, 2010).

In aqueous solutions, insulin is volatile. Hydrolytic reactions (deamidation) and polymerisation processes are the main causes of degradation.The primary deterioration consequence of low PH values is deamidation of asparagine amino acid in insulin chain A (A21), while deamidation of asparagine B3 occurs in neutral or alkaline media.Insulation-related compound A21 desamido insulin had a purity of less than 2%, while recombinant insulin purity must be greater than 98 percent; polymerization may also occur through transamidation reactions in neutral or alkaline media. The majority of industrial insulin formulations contain low concentrations of phenol and/or m-cresol as preservatives to prevent bacterial infection (Keating, 2012).

Insulin types, based on their mode of operation

At the basal stage, healthy humans produce insulin. After 1 hour of feeding, insulin sensitivity rises to a greater degree and drops to normal after another 2 hours. A single insulin solution with a specific action, peak effect time, and length of action cannot be used to achieve the normal 24-hour insulin profile and remove nocturnal hypoglycemia in diabetic patients. After being injected into the bloodstream, insulin molecules usually form hexamers (Chaluvaraju *et al.*, 2012). To expand the blood stream through the interstitial fluid and reach the capillary walls, these hexamers should be dissociated into dimers and monomers. As a result, different insulin formulations have been created to meet the needs of patients with different starting times, peak results, and activity periods. In these formulations, the dissociation rate of hexamers has been changed, as has the resulting movement of free insulin molecules into the bloodstream. The parts that follow explain the various forms of insulin formulations. Insulin Analogues from Ting (insulin glargine and insulin detemir) (Kalra and Kalra, 2004).

Acting Fast Insulin

The shorter lifetime and faster start time of these insulin analogs (15-30 minutes) are (4 to 5 h). Its maximum action, after injection, is 30-90 minutes. The ability of the insulin molecule to interact with the hexamer is diminished by one or two amino acid modifications, but these improvements have no effect on the analogues' physicochemical functions. Lispro and Aspart are two forms of rapid-acting insulin. Insulin Lispro reversed the structures of proline at position B28 and lysine at position B29 in the B chain. (LysB28, ProB29).

The proline in place was changed to aspartic acid in insulin Aspart. These fast-acting analogues can be used at mealtime to produce the most efficient insulin by using glucose released during intake (Wang *et al.*, 2012).

Insulin Short Acting

Short-acting insulin analogues take 0.5-1 hour to begin acting, peak action takes 2-4 hours, and the length of action is 6-8 hours.

Actrapid, Humulin, Hypurin, and Neutral are examples of these preparations. To achieve adequate insulin activity for carbohydrate digestion, these insulin analogues should be pumped into the body 20-30 minutes before the meal.

Intermediate Behaving with Insulin

The intermediate-acting insulin analogues took about 1-2 hours to start functioning, 6-10 hours to max out, and 10-16 hours to complete the surgery. NPH (Neutral Protamine Hagedorn) and LENTE are two intermediate-acting insulins (from the Latin lentus, which means slow or sluggish). The bioavailability of NPH insulin removes a need for protamine for insulin storage. The same will be needed for insulin LENTE if zinc is added to the insulin formulation.

Insulin with a Long Acting Time

These sugar analogues have a 2-hour onset of action, a 6- to 20-hour peak action period (sometimes without a peak action period), and a 36-hour operation period. Insulin analogues made from more positively charged amino acids have an isoelectric point of insulation near neutral pH, slowing glucose solubility even more after injecting into the bloodstream at neutral pH.To improve absorption, protamine or zinc have been added to some long-acting insulin preparations. Long-acting insulin detemir, also known as DesB30 Insulin, is an example. In insulin detemir, threonine is withdrawn from the B chain at position B30, and a 14-C fatty acid, myristic acid, is added to the lysine in the B chain at position B29. Insulin hexamer development is assisted by myristic acid, which enhances insulin attachment to plasma albumin, slowing insulin free release and increasing insulin activity.

Because of the minor variations in chemical structures, isolating insulin and its associated compounds is difficult. Many immune and nonimmune techniques for evaluating human insulin have been published. Human insulin has been identified in vivo and in vitro using radioimmunoassays, enzyme immunoassays, luminescent immunoassays,high-performance liquid chromatography (HPLC) and capillary electrophoresis Manufacturing and producing insulin analogues in the healthcare industry necessitates a technique with high precision and separation capability. As a result, achieving accurate analytical results requires selecting an appropriate analytical technique on use of insulin.The current insulin analysis techniques that would be appropriate for this research, growth, and clinical need for insulin are discussed in this report.

Insulin Determination Analytical Approaches

The various analytical methods for assessing glucose can be classified into three groups: 2. LC Methodologies 3. Immunoassays (Immunoassays) are a variant of 2.Electrochemical Methods. Immunoassays are covered by ELISA, AlphaLISA, HTRF, CLIA, and RIA. HPLC-UV, MECC, Bottom-up LC/MS, SPE-LC-MS/MS, Multidimensional LC-MS/MS, and Immunocapture-LC-MS/MS are the LC methods. Electrochemical biosensors are used in electrochemical processes (Figure 2).

Immunoassay

ELISA

Low-concentration proteins have long been studied and quantified using immunoassays. Although the immunoassay is versatile and responsive, its use in greater systems has been limited due to a lengthy process that requires multiple incubations and purification . The enzyme immunoassay (EIA) technique has grown rapidly in popularity over the last decade. Since it avoids the problems associated with the radioisotopic process, EIA has gained a lot of popularity as a substitute for radioimmunoassay (RIA) (such as special handling and disposal procedures, and limited reagent shelf life). Furthermore, since the enzyme mark may be detected at a single cell level, the EIA method may be more sensitive. Commercially available ELISA insulin kits had a lower limit of detection (LOD) of 3-12 pmol L-1 and a higher limit of detection (LOD) of 600 pmol L-1.29 The LODs were 0.73 IU mL-1 and 4.9 IU mL-1, with upper limits of 200 IU mL-1 and 324 IU mL-1, respectively (Abellan *et al.*, 2009). ELISA is suitable for research involving systemic salt and albumin solutions because it has a high degree of insulin selectivity and little interaction with other proteins. Some ELISA kits, however, showed a 54 percent cross-reactivity with intact proinsulin. There will be a clear need to improve insulin sensitivity as more advanced and special insulin trials are performed, that will be a major challenge for the widely used ELISA assays (Figure 3).

CLIA

Carslake *et al.* (2017) developed a CLIA assay method that is highly repeatable and appropriate for comparisons between other horses. The lowest possible dilution factor was used to dilute high-concentration samples with charcoal-stripped serum (CSS). At the amounts typically used to diagnose insulin dysregulation (roughly 100 lIU/mL). The CLIA demonstrated strong intra-assay variability (coefficient of variance [CV], 1.8-1).

Zhikun Zhan et al. developed an integrated polydimethysiloxane (PDMS) microfluidic system with two pneumatic micropumps and one micromixer for high-precision insulin detection. The method combines the highly precise "double-antibody sandwich immunoassay" with the sensitive LuminolHydrogen Peroxide chemiluminescence technique (H2O2).In

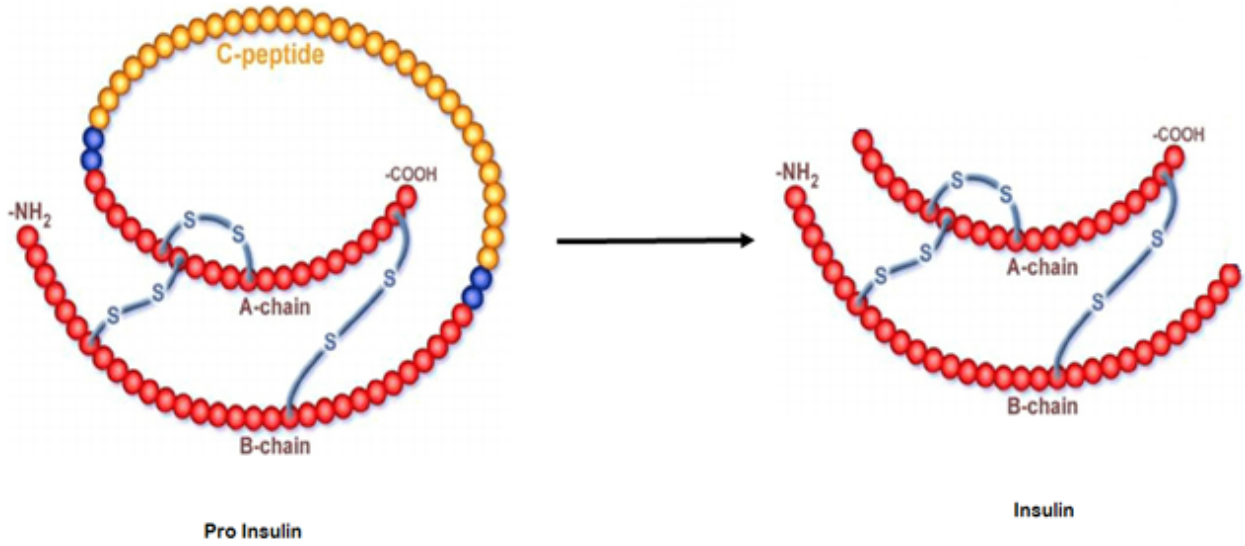

Figure 1: Conversion of Pro Insulin to insulin

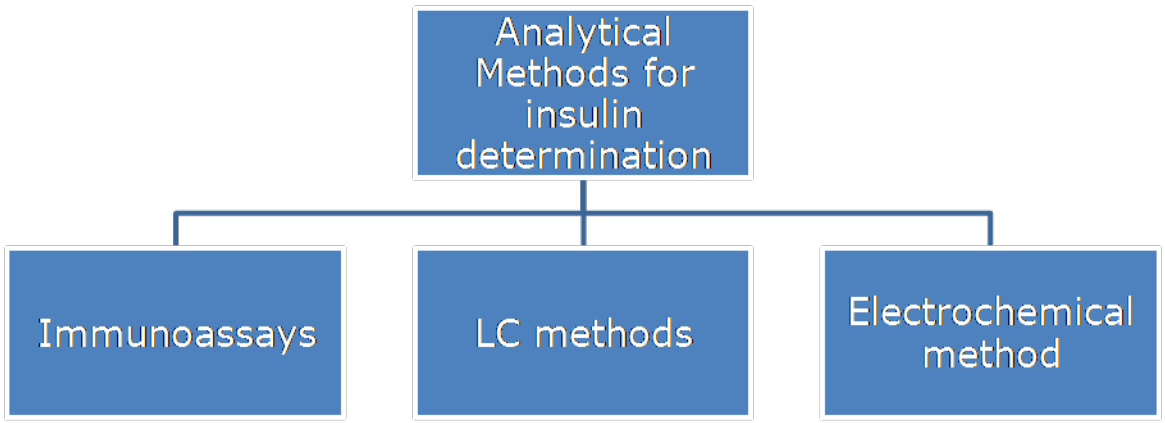

Figure 2: Analytical methods for insulin determination

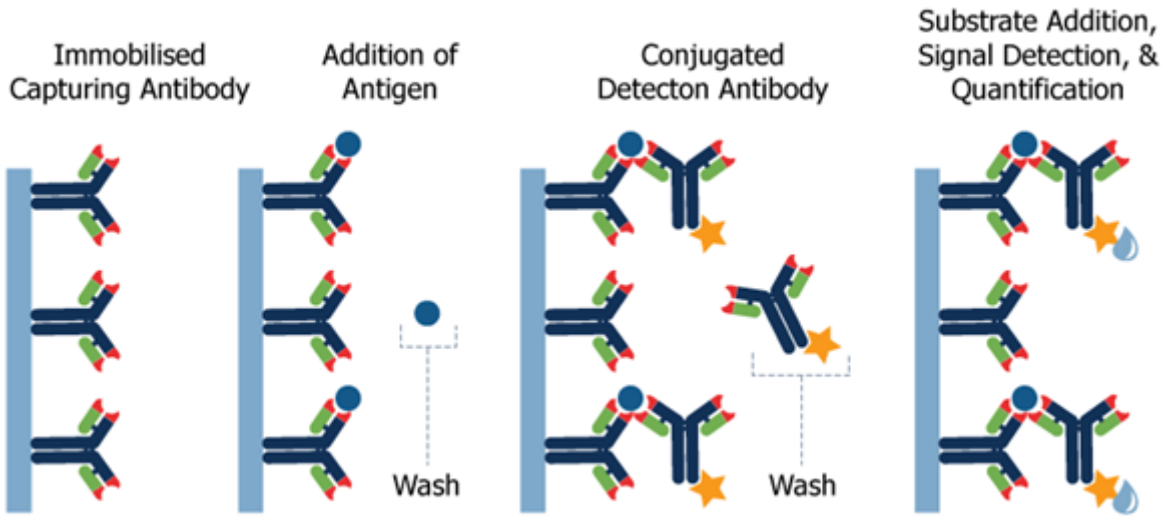

Figure 3: Direct Sandwich ELISA method (Courtesy: rockland-inc.com/elisa.aspx)

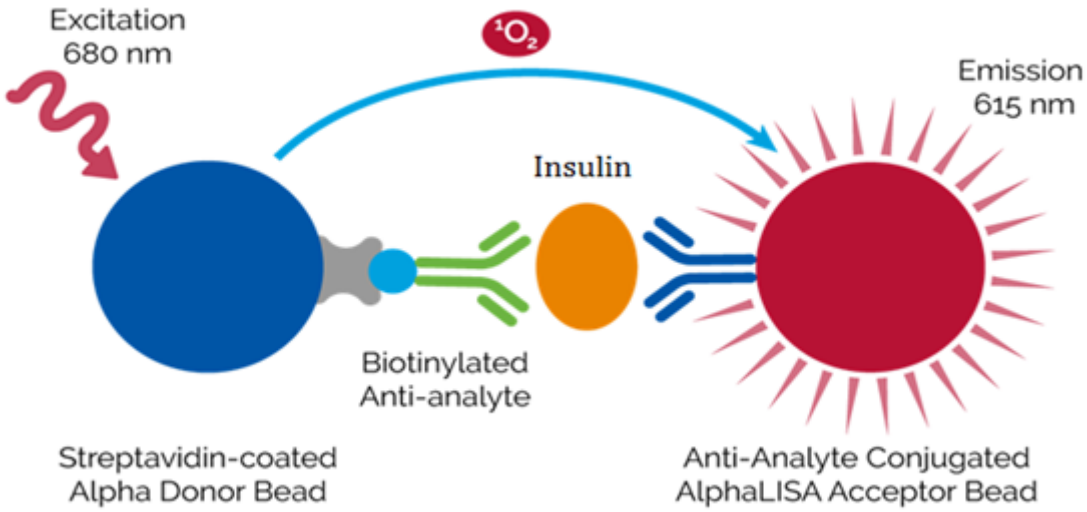

Figure 4: AlphaLISA assay (Courtesy: perkinelmer.com/product/alphalisa-hinsulin-kit)

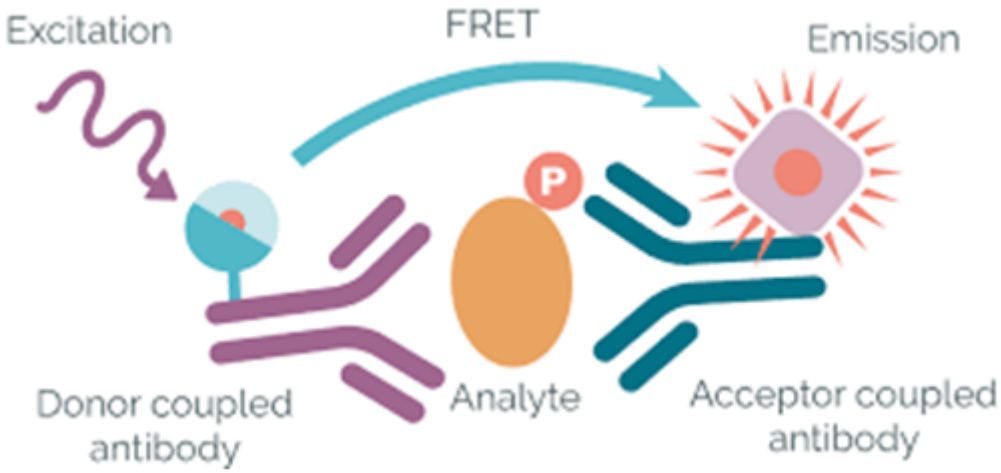

Figure 5: HTRF assay (Courtesy: cisbio.net/content/htrf-technology)

the microfluidic system, immune responses and other related processes are semi-automated. Two pneumatic PDMS micropumps are used in the microfluidic phase to move samples. Chemiluminescent tests are carried out in a separate PDMS micromixer fitted with a dual syringe pump for reagent administration. Because the chemiluminescent regents pass through the mixer chamber, the light produced by a mixer is measured by a highly sensitive photometer. The method has a few drawbacks, including low accuracy due to the lack of precise reference standards or equine insulin assays, and the need for more tests to standardise and validate assays.

AlphaLISA

Acceptor and contributor beads are coated with hydrogel in the AlphaLISA assay to create a bioconjugation functional group. Acceptor beads bind to a biotinylated anti-analyte antibody, while donor beads bind to another anti-analyte antibody coated with streptavidin. The biomolecular interaction in the nature of the analyte brings the beads closer together. The donor beads release singlet oxygen molecules, which triggers an energy transfer cascade also in donor beads, resulting in a sharp peak of light emission at 615 nm. Due to its vulnerability to ambient light leakage, photobleaching, andsinglet oxygen sequestration the system has decreased signal stability (Figure 4).

HTRF

The most widely used generic assay technology to quantify analytes in a homogeneous format is HTRF (Homogeneous Time Resolved Fluorescence), which is the optimal platform used in high-throughput screening for drug target studies. Fluorescence resonance energy transfer technique (FRET) is combined with time-resolved calculation in this technology (Georgiou *et al.*, 2014). The HTRF assay was successfully extended to all cell lines and pancreatic islets that secrete insulin and was found to be

Table 1: Immunoassays for estimation of Insulin

SL.No	Method	Advantages	Disadvantages	Author
1	ELISA	Selectivity is good; less interference; and high throughput	A few assays have cross-reactivity With intact proinsulin; not suitable for serum samples	(Manley *et al.*, 2000)
2	AlphaLISA	Higher-throughput screening ability	Signal strength is low.	(Rafati *et al.*, 2018)
3	CLIA	High signal intensity; absence of interfering emissions; wide dynamic range	Limited types of antigen identification; test panel and a higher cost	(Carslake *et al.*, 2017)
4	HTRF	Rapid and cost-effective; relatively low curve range	Not suitable for serum samples	(Farino *et al.*, 2016)
5	RIA	Large-scale use	Safety concerns; unspecificproinsulin, insulin, and insulin metabolite assessment	(Warnken *et al.*, 2016)
6	Chip based assay	Requires low amount of samples and bioreagents	Not willing to compromise for automation	(Wang *et al.*, 2001)

faster and more cost-effective. However, as serum can produce significant background fluorescence, serum insulin measurements are limited. Sample dilution will suppress background fluorescence, but this will decrease the system's sensitivity (Figure 5).

RIA

Within the RIA, a steady amount of glucose antibody must now be loaded onto test plate. There is competition between the two antibodies since a certain amount of radiolabelled antigen (125I) and unlabeled insulin samples are handled in the same well. When there is a greater ratio of unlabeled antigen, the antibody binds less radiolabeled antigen. 1.61 IU mL-1 to 200 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1.45 IU mL-1. The first commonly used insulin detection assay was the RIA, but it was difficult to use on a large scale. Other testing techniques, on the other hand, have gradually been phased out due to the need for a radiolabelled antigen and reagent volatility, both of which are safety issues. There are also drawbacks, such as non-specific confirmation of insulin precursors and products for insulin degradation alongside insulin.

Chip based immunoassays

Both enzyme and immunochemical tests are based on chip immuno assays. The immunochemical system is built into the biochip. Studies with enzymes in the same micro-channel. It makes simultaneous glucose analyses and the study is conducted using three stages of the pre-column, electrophoretic reaction isolation and reaction from the post column.In short, insulin immunometric tests, working with many assays on automated processes, are readily available. Yet the cross-reactivity of exogenous insulin was found in the determination of insulin analogs. For certain immunoassays that can detect insulin analogues, the total concentration of insulin and its analogues would also be the detected concentration (Table 1).

LC methods

When RP-HPLC is used to test insulin in commercial products, it can effectively isolate insulin from main degradation components like A21 desamido insulin and remove contamination from vial additives like

Table 2: LC methods for estimation for insulin

SL.No	Method	Advantages	Disadvantages	Author
1	HPLC-UV	Capable of distinguishing between insulin and its analogues	Long running time; requires baseline separation	(Yilmaz *et al.*, 2012)
2	MECC	Capable of distinguishing between insulin and its analogues	Requires baseline separation	(Lamalle *et al.*, 2015)
3	Bottom-up LC/MS	High-throughput and high specificity	Not Capable of distinguishing between insulin and its analogues	(Chen *et al.*, 2013)
4	SPE-LC-MS/MS	Capable of distinguishing between insulin and its analogues; not influenced by hemolysis	The SPE phase could cause interference from proteins with similar hydrophobicities to insulin.	(Darby *et al.*, 2001)
5	Multidimensional LC-MS/MS	Capable of distinguishing between insulin and its analogues	Complex sample pretreatment and specialised instrumentation are needed.	(Chambers *et al.*, 2014)
6	Immunocapture-LC-MS/MS	Capable of distinguishing between insulin and its analogues	The quantity of antibody and its affinity play a big role in sensitivity.	(Taylor *et al.*, 2016)

phenol or m-cresol. To differentiate between certain various insulin variants and insulins from different animals, researchers used a combined label-free full scan technique.

Their method was also able to chromatography distinguish human insulin and insulin lispro in conditions that were consistent with mass spectrometry (MS). Using the International Community's validity criterion, the researchers were also able to measure different insulins selectively, including human insulin and insulin lispro.

While several scientists noticed to use HPLC to determine insulin content as a simple tool for protein purity determination, due to poor chromatographic conditions, The majority of those tests were unable to distinguish among insulin and its desamido type with sufficient precision. The majority of these approaches have used a low pH and high salinity mobile step to try to regulate the degree of insulin ionisation, but this has affected column stress and resulted in process variations. Other techniques used the gradient mobile step, but due to the long retention period, these were found to be time consuming and unsuitable for routine analysis (20-45 minutes). Another drawback of the published methods is that they necessitate the use of an ion-pairing reagent and column temperature controller both of which are uncommon in most analytical laboratories.

LC-MS/MS is commonly used in biological matrices for the analysis to therapeutic proteins/peptides and biomarkers. The LC-MS/MS is superior because of its extreme precision and selectivity, as well as its high dynamic range and resistance to interference (Table 2).

Electrochemical methods

Among biosensing technologies, electrochemical techniques have been commonly used to capture events of biological recognition and transform findings directly into the field of electronic content. In efficiency and usefulness, electrochemical biosensors have many advantages relative to conventional optical biosensors:Many offer label-free identification, reducing the time, expense and difficulty of usage associated with sample marking methods, and are ideally suited to wearable platforms, for example, for miniaturization. The carbon fibre microvoltammetric electrode modified with polynuclear ruthenium oxide/cyanoruthenate film has been documented by Schmudlach *et al.* (2016).

For insulin estimation, with a detection limit of 0.5μMM. The insulin aptamer is covalently con-

nected to an electrochemical sensor made up of carbon nanotubes (CNT). In a three-electrode unit, the developed insulin-aptamer biosensor serves as the operating electrode, with Ag/AgCl as the reference electrode and a platinum wire as the counter electrode. The recognition event is converted into the clear and readable signal by the signal transducer. Using cyclic voltammetry with a potential range of 1 to 1 V and a scanning speed of 0.1 v s1, insulin's electrochemical signal was calculated.To increase the sensitivity of the insulin detection process, carbon ceramic electrodes were produced using the sol-gel technique.

The electrical biosensor device has the following advantages: its simplicity, high accuracy, minimal instrumentation and complicated labeling techniques are avoided. The sensitivity of this insulin technique, with exception of the receptor-ligand pair, is surprisingly low, since only a trace of unknown bound bovine serum albumin could be found.

CONCLUSION

Immunoassays, chromatographic assays and novel science, commercial, and research biosensor assays were all investigated in this study. Because of its high output, ELISA has long been a standard instrument in therapeutic immunoassays. Modern analytical platforms, such as mass spectrometry techniques, on the other hand, are high-sensitivity and hopeful instruments that can distinguish among insulin and its analogues. In general, modern insulin analytical methods will continue to concentrate on simplifying sample preparation while keeping processing times short and costs low.

ACKNOWLEDGEMENTS

The authors are grateful for the supply of research facilities by the management of Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai-600 117, Tamil Nadu, India.

Conflict of Interest

The authors declare that there is no conflict of interest for this study.

Funding Support

The authors declare that they have no funding support for this study.

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