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Biological Assay of Insulin: An Old Problem Re-Discovered

Alaaeldin Ahmed Hamza*

Department of hormone Evaluation, National Organization for Drug control and Research, Egypt

***Corresponding author:** Alaaeldin Ahmed Hamza, Department of hormone Evaluation, National Organization for Drug control and Research,6 Abu Hazemst, Giza, Madcor, Egypt, Tel no: (+002) 01147739950; Email: alaaeldin.hamza@hotmail.com; alaa17mm@gmail.com

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Abstract

Bioassays are methods employed to estimate the effect of a given substance in living matter, and therefore they are frequently used in the pharmaceutical industry. Bioassays are a vital part of the overall assessment of a product's efficacy, safety, lotto lot consistency and stability. These more complex entities can challenge the developer of the bioassay to generate potency assay that truly mimic the mechanisms of activity. Strategies and challenges for bioassays for insulin will be discussed during this review. The European Pharmacopeia Commission now supports a program of research to replace animal-based testing, and to delete the requirement for animal testing to assess the efficiencies of insulin. Although insulin (51 amino acids, molecular mass of 5800) are now regarded as chemical drugs, dose in milligrams, and approved for use in human without the need for any form of bioassay. The European Pharmacopeia does not require such an in vivo bioidentity test. However, the animal testing is still required in United States Pharmacopeia to assay the efficiency of insulin. The United States Pharmacopeia mandates an animal based assay in rabbits in its chapter "Insulin Assays" for the potency evaluation of insulin and insulin analogs to be imported to the US. There are argent needs to review and discuss this issue.

Keywords: Insulin; Biological assay; Biological medicine; Physical–chemical method

Abbreviations: FDA: Food and Drug Administration; HPSEC: High Pressure Size Excision Chromatography; NMR: Nuclear Magnetic Resonance; COD: Cross-over design.

Introduction

As ongoing advances in biochemical and biophysical characterization continue to illuminate the molecular features of biological molecules, the measurement of biological potency will help establish structure–function correlations, assist in determining of immunologic response, and elucidate the molecule's biological identity. The science of biological standardization is actually divided into three disciplines: development of quantitative biological assay systems, in animals, organs, cells and sub-cellular fractions, the development of statistical methods to analyse such assay systems, and the development of procedures to prepare, store and test reference materials, solving problems of scale, retention of activity, stability and homogeneity [1]. Most importantly, stability in potency results assures practitioners and patients that they are receiving a product with consistency in anticipated therapeutic

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outcomes batch-after-batch over many years following market access [2]. Bioassays are a vital part of the overall assessment of a product's efficacy, safety, lotto lot consistency and stability. These more complex entities can challenge the developer of the bioassay to generate potency assay [3] that truly mimic the mechanisms of activity. Strategies and challenges for bioassays for Insulin will be discussed during this review. The European Pharmacopeia Commission now supports a program of research to replace animal-based testing, and to delete the requirement for animal testing to assess the efficiencies of insulin. However, the animal testing is still required in USA to assay the efficiency of insulin [4,5]. There are argent needs to review and discuss this issue.

Insulin as Biological Medicines: Their Origin and Definition

Paradoxically, however, insulin available in the early decades of the twentieth century was extremely impure, and determination of content/potency using modern analytical methods would have been quite impossible. Moreover, its structure was unknown until the 1950s, when Sanger elucidated it in his Nobel prize winning protein structure studies. In the absence of any meaningful knowledge of the structure or analytical methods to measure the content, the dose of insulin to be administered was determined by quantitative measurement of its biological activity [1].

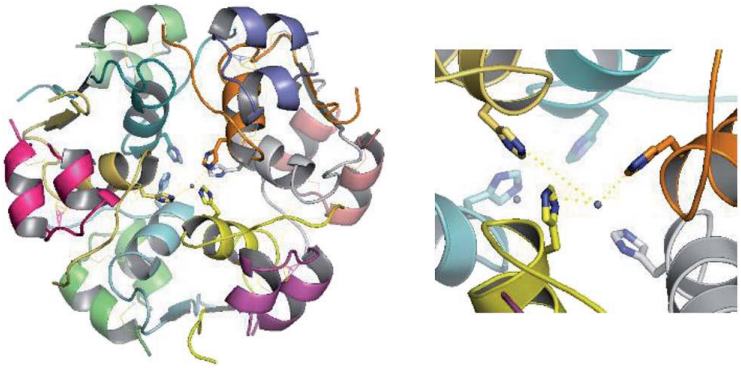

Figure1: Insulin structure. Left: Three dimers aggregate around 2 Zn^{2+} ions in hexamerformation. Each chain of the insulin hexamer is represented with a different color. Right: Coordination between a Zn^{2+} ion and three His (B10) side chains from [6].

Evolution of Insulin: From Animal to Analogs

The history of insulin therapy has been one of continually evolving improvement. Since insulin was first isolated in 1921, milestones in this evolution have included the development of a slower-acting preparation in the late 1940s, neutral protamine Hagedorn insulin, and the use of recombinant technology to enable production large amounts of insulin. The pioneering work of Stanley Cohen and Herbert Boyer, who invented the technique of DNA cloning, signaled the birth of genetic engineering, which allowed genes to transfer among different biological species with ease [7]. Their discovery led to the development of several recombinant proteins with therapeutic applications such as insulin and growth hormone. Genes encoding human insulin and growth hormone were cloned and expressed in E. coli in 1978 and 1979 respectively. Although chemically synthesized human insulin was first produced in the 1960 and studied in preliminary clinical trials, this breakthrough was overshadowed by the advent of recombinant DNA technology in the late 1970s, which gave rise to recombinant human insulin in 1978 [8]. The first licensed drug produced using recombinant DNA technology was human insulin, which was developed by Genentech and licensed as well as marketed by Eli Lilly in 1982 [3]. This synthetic insulin was named "human insulin" to distinguish it from the earlier preparations derived from animal sources. Further milestones were the introduction of rapid-acting insulin analogs in the 1990s and long-acting basal analogs in the early 2000s [9].

Until the 1980s animal insulins, extracted from either bovine or porcine pancreata, comprised all commercially available insulin formulations. Since the 1980s, insulin replacement strategies have been incrementally optimized, with the development of structurally modified, so-called designer insulin analogues and the first bioengineered commercial insulin analog (Lispro) in 1996 [8]. Currently, widely used basal insulins are the intermediate acting neutral protamine Hagedorn insulin and the basal analogs insulin glargine (Lantus; Sanofi, Paris, France) and insulin detemir (Levemir; Novo Nordisk Inc, Plainsboro, NJ). One additional analog,

insulin degludec (Tresiba; Novo Nordisk Inc), has been licensed for use in Europe and Elsewhere. Insulin degludec, licensed in Europe and Japan but not yet in the United States, has a mean half-life of 25.4 hours, a duration of action of >42 hours [9] (Figure 2).

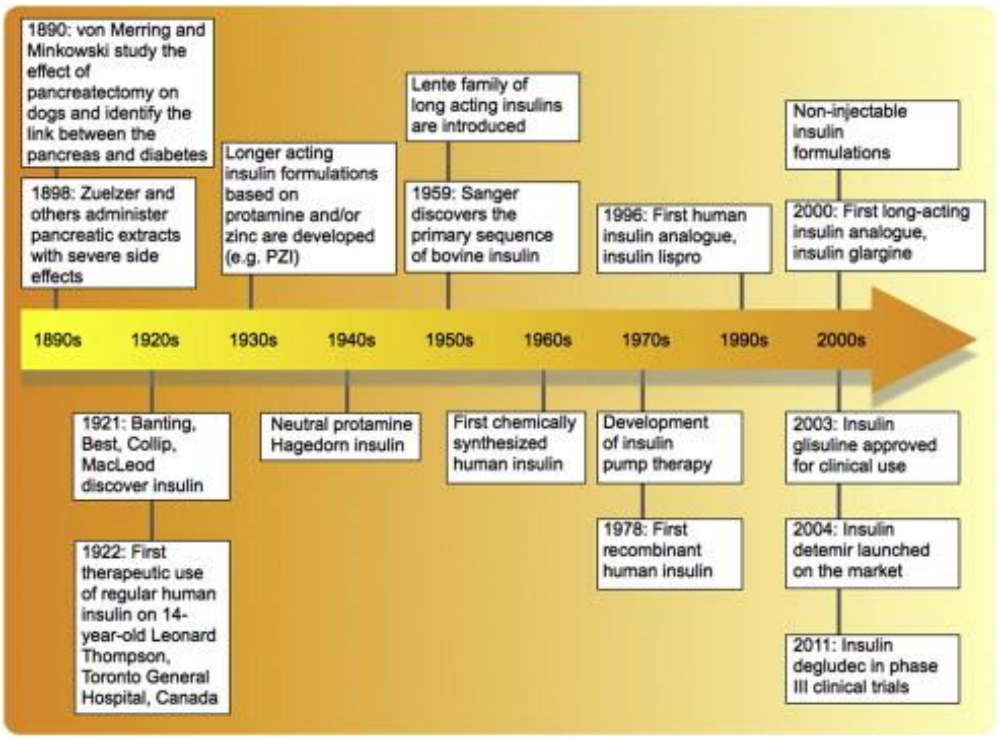

Figure 2: Selected evolutionary milestones in insulin replacement therapy from [8].

Biological Assays

Biological assays or "bioassays "are a set of techniques for estimating the potency or strength of an "agent" or "stimulus" by utilizing the "response" or "effect" or "reaction" caused by its application to biological material or experimental living "subjects [10].

Standard

It usually refers to a batch of samples of a reference material - a 'biological standard' -with which other preparations of similar material are quantitatively compared. A measured amount of a biological standard is assigned to define a quantity of that activity, in terms of a number of 'units of biological activity'. The biological 'unit' expressed in an assay system quantifies activity not mass.

Importance of Bioassays

Bioassays, as compared to other methods of assays (e.g. chemical or physical assay) are less accurate, less elaborate, more laborious, more troublesome and more expensive. However, bioassay is the only method for release of drug substance (active pharmaceutical ingredient) and drug product; stability; qualification of standard sample and other critical reagents; characterization of process intermediates and formulations, contaminants, and degradation products; and support of changes in the product production process [5].The determination of biological potency plays a key role in the development, registration, and control of biological and biotechnology-derived products.

Dose the Change in Blood Glucose Level of Animal is a Biological Assay of Insulin?

Blood glucose-lowering effect in animal such as rat, rabbit and mouse has been chosen for primary screening for blood lowering compound as well as for establishing time-response curve [11]. The biological assay is an experiment in which interest lies in comparing the potencies of the treatment on agreed scale and different from traditional comparative experiments. Thus an investigation into

effects of different samples of insulin on blood sugar of rabbits is not necessary a biological assay. It becomes one if the interest lies not simply in the change in blood glucose levels, but in their use for the estimation of the sample on the scale of standard units of insulin [10,12].

In Vivo bioassays

In vivo potency assays are bioassays in which a set of dilutions of each of the standard and test materials is administered to animals and the dose-response relationships are used to estimate potency. For some animal assays, the end point is simple (e.g., rat body weight gain assay for human growth hormone or rat ovarian weight assay for follicle stimulating hormone), but others require further processing of samples collected from treated animals (e.g., reticulocyte count for erythropoeitin, steroid genesis for gonadotropins, neutrophil count for granulocyte colony stimulating factor, or antibody titer after administration of vaccines) [5]. Following the discovery of insulin in 1921, biological assays developed to assess the potency of the hormone, were based on the measurement of the hypoglycemic response in rabbits and Convulsive response in mice[1,5].

Principle of bioassay

The basic principle of bioassay is to compare the test substance with the international standard preparation of the same and to find out how much test substance is required to produce the same biological effect, as produced by the standard [12]. The potency assay should not be too complex or difficult and should be suitable for routine performance in the quality control laboratory. The assay should be highly accurate, precise, specific to the target, and stability indicating. Accurate quantification of the active principle was achieved by quantifying its activity in a test measuring function rather than quantity of substance, i.e. what it does, rather than what it is, and quantification is dependent on the science of biological standardization. the unit of insulin can only be described in terms of a reference material, not in terms of an absolute response (e.g. number of convulsions), and statistical combination of independent assays can produce precise numbers from an inherently imprecise and irreproducible method. Valid measurement of a biological activity by comparison [3]of a test sample with a quantity of a biological standard depends on certain fundamental assumptions.

a. Firstly, that the material in both test and standard are the same or at least that the test sample behaves in the bioassay as though it was a dilution of the standard.
b. Secondly, that the effect which is measured as the response in the test system, represents a true and relevant property of the intended analyte in the materials compared.
c. Thirdly, that the doses of test and standard are compared by means of an appropriate and validly randomized experimental design in the assay system [12].

History of biological assay of insulin

During its early history, while chemical and physical analytic procedures cannot completely characterize the molecular substance, the identity of certain hormones is virtually 'defined' by the reference material and the particular assay method used to quantify activity.After production of insulin started in '23, great efforts were made to improve bioassay methods and the statistical procedures to evaluate the results. The bioassay first used for insulin was the rabbit blood glucose method which involved measurement of the drop in blood glucose concentration in rabbits at half hourly intervals after injection of a dose[13]. A biological assay of insulin preparations in comparison with a stable standard using the blood sugar lowering effect in rabbits has been proposed already in 1925 by Harrison et al. The biological assay of insulin using the blood sugar lowering effect in rabbits has been until recently the official assay in several pharmacopoeias, such as European Pharmacopoeia, British Pharmacopoeia, United States Pharmacopoeia [14] and The National Formulary. The current USP test for bioidentity is the in vivo rabbit blood sugar bioidentity test [15].

The biological assay of insulin using hypoglycaemic seizures in mice has been suggested already in 1923 by Fraser. The biological standardization of insulin using the mouse convulsion method has been published in detail by the Health Organization of the League of Nations in 1926 and has been until recently the official assay in several pharmacopoeias, such as European Pharmacopeia, British Pharmacopoeia[16]. In most pharmacopoeias, the biological assays have been replaced by chemical methods (British Pharmacopoeia 1999; European Pharmacopoeia) [15]. Groups of mice, each injected with doses graded in units of either test product or of insulin standard, were closely observed and the number in each group that developed hypoglycemic convulsions was counted. In time it was realized that the threshold for indications of hypoglycemia in mice was exquisitely sensitive to many factors, including strain, season, diet, temperature, noise, handling and number of mice per container [13,17].

Eneroth & Ahlund[18]recommended a twin crossover method for bio-assay of insulin using blood glucose levels in mice instead of hypoglycaemic seizures giving more precise results. This was made possible by the availability

of an Auto Analyser micro-method [19] for the measurement of blood glucose, thus enabling a major advance in precision and reliability. Comparisons between the mouse blood glucose and mouse convulsion methods were favorable and the advantages of the blood glucose method, such as reductions in the number of mice used and reduced mortality made the change well worthwhile. All such assays were comparison assays, comparing the 'strength' of measured quantities of a batch under test, with measured quantities of the relevant biological standard. All of them gave relatively imprecise results [13].

What's a Good Strategy for Developing Robust Bioassay Systems?

Reduction in bioassay variability

The problem of biological variation must be minimized as far as possible. For that one should keep uniform experimental conditions and assure the reproducibility of the responses [12].

Experimental design

Virtually all animal experiments should be done using one of the formal designs described in Pharmacopeia. In principle, a well-designed experiment avoids bias and is sufficiently powerful to be able to detect effects likely to be of biological importance. It should not be so complicated that mistakes are made in its execution. Virtually all animal experiments should be done using one of the formal designs described briefly below [20].

Completely randomized design

If the totality of experimental units appears to be reasonably homogeneous with no indication that variability in response will be smaller within certain recognizable sub-groups, the allocation of the units to the different treatments should be made randomly.

The parallel-line model

A parallel line assay in which each of the preparations has an equal number of doses and an equal number of subjects is allotted to each of the doses, is called a symmetrical parallel line assay. Example is an assay of insulin by hypoglycemic seizures method.

Cross-over design (COD)

Blood glucose determination in rabbits and mice depend on a twin crossover method for bio-assay of insulin. This design is useful when the experiment can be sub-divided into blocks but it is possible to apply only 2 treatments to each block. For example, a block may be a single unit that can be tested on 2 occasions. The design is intended to increase precision by eliminating the effects of differences between units while balancing the effect of any difference between general levels of response at the 2 occasions. If 2 doses of a standard and of an unknown preparation are tested, this is known as a twin cross-over test. The experiment is divided into 2 parts separated by a suitable time interval. Units are divided into 4 groups and each group receives 1 of the 4 treatments in the first part of the test. Units that received one preparation in the first part of the test receive the other preparation on the second occasion, and units receiving small doses in one part of the test receive large doses in the other [21].

Does Data Processing (I.E. Parallel Line Vs Cross Design) Play a Role in Method Precision?

In COD, the same subject serves as both the test and control arms which allows for within-subject comparison. Hence, approximately half the sample size is required to yield the same statistical power as a two arm parallel design. This minimizes the cost involved in the recruitment and clinical investigation process. Also, within-subject variability is relatively small compared with between-subject variability in parallel group design. Because of this small variability coupled with the small sample size, there is a high level of precision associated with point estimates. On the other hand, potential bias is brought to a minimum in COD. Randomization and comparison within the same participant serving as both the reference and the test intervention allows exposure to the same conditions unlike between subjects where one participant differs both internally and externally from another [21].

Critical assessment of those *in vivo* biological methods

a. As with many experiments with animals the precision obtained with such hormone bioassays depended very much on the quality and uniformity of animals used: on their being housed in suitable groups, fed on a standardized diet, and kept in conditions with minimal disturbance, conditions which demanded considerable expertise and care in the animal house. All such assays were comparison assays, comparing the 'strength' of measured quantities of a batch under test, with measured quantities of the relevant biological standard. All of them gave relatively imprecise results.
b. Blood glucose determination in rabbits and mice depend on a twin crossover method for bio-assay of insulin. The rabbit bioidentical test is characterized by substantial analytical variation and the frequency of re-

runs due to failure to meet statistical a requirement was approximately 20%.

c. Science 1986 Animal Act in Britain, arguments have been forward against the use of retro-orbital plexus as a source of the 50µl of blood needed to measure the hypoglycemic response of mice. The argument is based on the supposition that the retro-orbital plexus is not a superficial blood vessel and such should only be accessed with the animal under anesthetics [22]. Unfortunately, the use an anesthetic could well affect the mouse's apparent response to insulin. This test was inducted into the British Pharmacopoeia 1980 and continued up to 1988 [15].
d. The shortcoming of hypoglycemic seizures method needs ninety-six mice of either sex for every assay.

Replacement of *in vivo* bioassay test with the cell –based bio identity test

Advances in recombinant DNA technology and the understanding of cellular signaling mechanisms have allowed the generation of engineered cell lines with improved response and longer stability [5]. The recently developed in vitro cell-based bioidentity test is suitable for measurement of the bioidentity of insulin human and insulin as part and is based on genetically modified cell culture. Consequently, Novo Nordisk has requested Food and Drug Administration (FDA) to replace the method of analysis for bioidentity to the insulin as part and insulin human drug substance specifications with the cell-based in vitro method of analysis. A replacement of the rabbit method is in accordance with the decision by the Environmental and Bioethics Committee at Novo Nordisk A/S to remove all internal and external requirements for the use of living animal for product control by 2010, if at all possible. It is also in accordance with Interagency Coordinating Committee on the Validation of Alternative Methods Authorization act of 2000 that enacts to reduce, refine or replace animal testing [23,24].

Sanofi started a project to validate an experimental in vitro test as an alternative to the rabbit bioidentity assay with two objectives [24]. The first objective was to enable the export of drugs containing insulin glargine to the US without animal experiments and the second objective was to achieve the inclusion of the in vitro method as an alternative to the rabbit bioidentity assay in the USP for all insulins for the US market. As a result of Sanofi's groundwork, USP recently published a document for comment in section "General Chapters, In-Process Revision: <121> Insulin Assays" [5], stating "a new in vitro cell-based bioidentity test for Insulin Glargine and Insulin Lispro is added as an alternative approach to in vivo bioidentity test using rabbits. The new test was validated and approved by the FDA for assuring potency of a particular manufacturer's approved products. In the future, USP plans to omit the rabbit method completely after multiple manufacturers have been able to demonstrate that the cell-based method is appropriate for all their insulin products. This is part of USP's ongoing effort to phase out animal-based assays in favor of modern in vitro testing."

Replacement of *in vivo* bioassay test with physical–chemical methods

An estimation of potency derived from a biological assay is subject to random error due to the inherent variability of biological responses and calculation of error should be made from the result of each assay even when the official method of assay is used [25]. From the start it was understood that when the active substance of a biological substance becomes readily purified, characterized, identified and quantified, the need for a biological standard for it ceases and the standard is formally discontinued. In this context, several physical–chemical methods have been reported in the literature as potential alternative assays for determining the biological activity of different proteins with greater precision and accuracy. As a case in point, size-exclusion high performance liquid chromatography (HPSEC) was shown to be adequate for the determination of the potency of insulin.

Current Pharmacopeial Situation and Future Trends

Potency is the property of the drug, not of the response. Protein therapeutics, including insulin, are a class of products which have a complex three dimensional structure in solution whose integrity determines the biological activity, clinical efficacy, and safety. Thus, it is highly desirable that products from this class meet well-defined requirements for structural integrity. Proteolytic footprint has long been the method of choice, generating a peptide mapping which indirectly provides evidences for solution structural arrangement [26]. Several techniques are currently in use for identity confirmation of Pharmacopeial biological drug substances. For the vast majority of these compounds, this includes LC-UV analysis based on retention of intact and/or digested peptide by comparison with reference material. In the Ph. Eur. the retention time-based specificity is further increased by amino acid analysis, and spectrometric techniques e.g. IR, mass spectrometry [6], nuclear magnetic resonance (NMR) and UV. NMR spectroscopy can provide unique information about peptide structure, dynamics, hydration and folding in the solution state [6,26].

At the present time, physical-chemical analytical methods address the structural complexities, and inherent

heterogeneity of insulin products. These combined methods can assist in the determination of chemical integrity (mass spectrometry), solution conformation (NMR fingerprint), and structural integrity (NMR and X-ray crystallography) of regular acting human insulin in final formulations [6,26]. However, to date no high-resolution structural requirement is made for insulin in the current US, European, Japanese, and Brazilian pharmacopeias. Instead, the biological and, indirectly, the structural integrity of insulin are indirectly accessed through biological assays, with the single-point evaluation of the glycemia lowering efficiency by insulin products, along with other biochemical proofs of purity and potency. USP plans to omit the rabbit method completely after multiple manufacturers have been able to demonstrate that the cell-based method is appropriate for all their insulin products. These physical-chemical methods are currently well-established, and they can be accessed in most countries, in special those for the main pharmaceutical markets, the Americas, Europe and Japan, whose pharmacopeias were above mentioned. It could be used in routine evaluation of structural integrity and identity, as a part of current or evolving methods aiming the minimization of animals' requirement in routine quality control, in the development of novel insulin products, or in future protocols for a thorough comparability exercises between follow-on protein product and a reference product. I believe that the information gathered in the present study would assist in further establishment of biological drugs for routine pharmacopeical and metrological quality analysis.

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