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Bio-assays for the analysis of insulin*

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Abstract: An account is given of the development of tests for the bio-assay of insulin and the progress made, at the Wellcome Foundation Limited, during the last 30 years in reducing the number of animals used in the testing of insulin for the treatment of Diabetes Mellitus. The progressive evolution of methods shows how the number of mice used per sample has already been reduced five-fold and an ongoing development of *in vitro* methods, particularly high-performance liquid chromatography (HPLC), has enabled Wellcome to recommend to the regulatory authorities alternative procedures to replace animal testing.

Keywords: *Insulin bio-assay; mouse convulsion method BP; mouse blood glucose method BP; radioimmunoassay; radioreceptor assay; high-performance liquid chromatography.*

Introduction

Following the discovery of insulin in 1921 [1], because of lack of knowledge of its chemical structure, biological assays were developed to assess the potency of the hormone based on the measurement of the hypoglycaemic response in rabbits and convulsive response in mice. Variations of these approaches have been used since to control the potency of insulin in pharmaceutical preparations. When Banting and Best and their co-workers [2] found that insulin induced convulsions in rabbits were usually associated with low blood glucose levels, the amount of insulin which produced convulsions in a rabbit in 2–4 h was defined as a unit of activity. Later in 1925, Marks [3] recommended the comparison of blood sugar estimates in rabbits using a "test" preparation of unknown activity in terms of another reference preparation of known activity, with the procedure being reversed on a separate day to complete a "cross-over" test. In 1936, Marks and Pak [4] proposed a further modification in that two dose levels of the reference and test preparations were tested in twin "cross-over" experiments to provide a better estimation of the potency. This broadly formed the basis of the assay method using rabbits adopted by the United States Pharmacopeia [5].

In 1926, Hemmingsen and Krogh [6] showed that the percentage of mice which responded to insulin by convulsion became greater with increasing dose. A quantal assay based on this convulsive technique was reported by Trevan and Boock of the Wellcome Foundation in 1926 [7] and is still retained after a number of refinements in experimental

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design and analysis, as one of the official methods of the most recent editions of the British and European Pharmacopoeias.

Developments in bio-assays for insulin

The Wellcome Foundation at Dartford, UK, made use of the quantal mouse convulsion method for the assay of insulin from its inception until 1978 while pressing for the inclusion of a quantitative test, the mouse blood glucose method, in the British Pharmacopoeia. The conversion to the mouse blood glucose method of Eneroth and Ahlund [8] then coincided with its introduction into the British Pharmacopoeia [9]. This was made possible by the availability of an AutoAnalyser micro-method [10] 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 favourable 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.

This change to the mouse blood glucose assay for insulin allowed a reduction in the average number of mice used per sample representing a batch from 600 to 130. With repetitive use of each animal, up to 16 times with a week's rest in between, and with improvements in production technology which led to larger batch sizes of formulations, a further reduction in the number of mice used was achieved.

Thus progress to date has resulted in the situation where the use of one mouse for the assay of insulin is sufficient to control the total insulin requirements of one diabetic person for the whole of his lifetime treatment. Figure 1 shows the number of mice used per 1,000,000 units of insulin sold by the Wellcome Foundation over the period 1970–1986 and demonstrates how it has been possible to make progressive reductions in the number of mice used since 1970. However, since the 1986 Animals (Scientific Procedures) Act in the UK, arguments have been put forward against the use of the retro-orbital plexus as a source of the 50 μl of blood needed to measure the hypoglycaemic response of mice. The arguments are based on the supposition that the retro-orbital plexus is not a superficial blood vessel and as such should only be accessed with the animal under anaesthetic. Unfortunately, the use of an anaesthetic could well affect the mouse's apparent response to insulin and some development work would be necessary before the technique could be changed.

With regard to the repetitive usage of mice, Fig. 2 demonstrates that the results of mouse blood glucose insulin assays obtained by the use of naive or once-used mice have lower statistical weights than those generated using mice which have been used between 2 and 16 times with, of course, appropriate resting in between tests. This is possibly because the naive mice, which are not accustomed to being handled, secrete varying amounts of anti-insulin hormones. This theory is supported by the fact that it is necessary to administer larger doses of insulin to naive mice. This improvement in statistical weight with times of use is not seen in the mouse convulsion test, probably because of the higher dose needed to cause a convulsion.

Improvements in sensitivity

The mouse convulsion and the rabbit and mouse blood glucose methods are sufficiently sensitive to detect insulin at the concentrations found in pharmaceutical

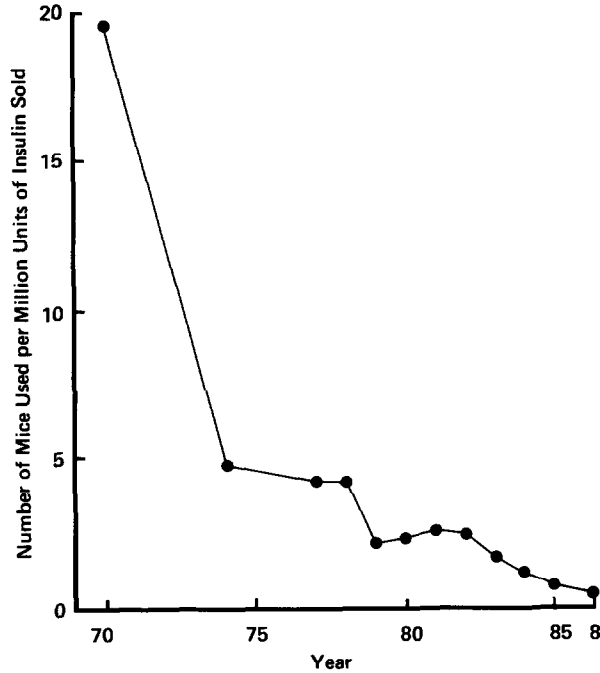

Figure 1
Reduction of animal usage for insulin assay at the Wellcome Foundation during the period 1970–1986.

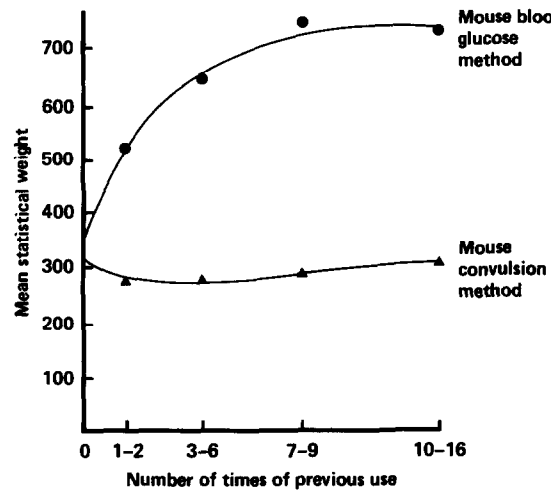

Figure 2
Assay of insulin formulations — statistical weight compared with the number of times of previous use of mice for both mouse convulsion and mouse blood glucose methods.

preparations. Although they are the only currently internationally accepted assay methods, many other bio-assays for the analysis of insulin have been developed over the years for research and assay purposes.

Among the more sensitive is an assay developed by Bornstein in 1950 [11] in which adrenalectomised, hypophysectomised rats rendered alloxan diabetic were demonstrated to be sensitive to as little as 50 micro-units of insulin, whereas a normal intact mouse is sensitive to only 200 times that amount. However, the skilled surgery required for this Bornstein technique makes it generally unsuitable for routine insulin assays.

About the same time, fairly sensitive *in vitro* methods were developed for the assay of insulin. It was shown by Groen *et al.* in 1952 [12] that there is a relationship between insulin concentration and glucose uptake in the rat diaphragm. This method is sensitive to about 10 micro-units of insulin. Another method was developed by Winegrad and co-workers in 1958 [13] based on the glucose uptake by the rat epididymal fat pad, which was sensitive to 30 micro-units of insulin. However, according to Wright in 1960 [14], several substances, for example, free fatty acids and adrenalin, interfere with the diaphragm method and it was further shown by Riesler in 1967 [15] that proteolytic enzymes leak out of the cut muscle fibres to degrade the insulin. Also several authors have queried the specificity of the fat pad assay [16, 17]. So in spite of being very sensitive to insulin, both types of *in vitro* assay lacked precision and specificity.

Radioimmunoassay procedures

In 1960, Yalow and Berson [18] devised a sensitive, precise and specific radioimmunoassay for the determination of insulin at physiological concentrations. In 1965, the radioimmunoassay technique of Hales and Randle [19] was examined in Wellcome's Laboratories where a large number of insulin stability samples was considered to be the ideal proving ground for this method (Fig. 3). Unfortunately, the sites on the insulin molecule which act as immunogenic determinants are not the same as those involved in

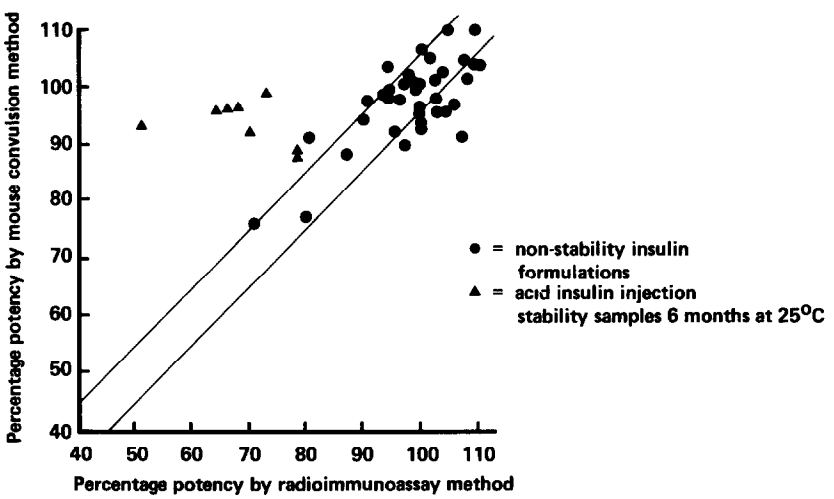

Figure 3
Assay of insulin formulations — comparison of mouse convulsion and radioimmunoassay methods for both stability and non-stability insulin formulations.

binding the biological receptors which mediate the action *in vivo*. Therefore results obtained were disappointing as they did not always correlate with biological potencies obtained for the same samples. It was also suspected that thermally induced changes in the configuration of the insulin molecule were causing some changes in molecular conformation, such as mono- or di-desamidation, leading to immunoassay values which in Wellcome's hands were falsely low or in other hands were falsely high, depending on the antibody used. As the radioimmunoassay could not be shown to correlate with clinical or *in vitro* efficacy, and because of the real difficulty of reagent standardisation, it was not considered suitable for adoption by the regulatory authorities.

However, the radioimmunoassay technique has been regularly used for fresh samples to monitor insulin activity during manufacture and purification. This technique costs about one sixth of the current *in vivo* bio-assay method and has the added advantage of not using animals. Therefore it is the method of choice for in-process samples.

Other insulin orientated radioimmunoassay techniques have also been utilised at Wellcome to determine the amounts of hormone impurities, such as proinsulin, glucagon and pancreatic polypeptides, in order to ensure that they are present only in the lowest concentration possible in the finished product.

Radioreceptor assay

Disappointments in the radioimmunoassay technique for the measurement of insulin potency led Wellcome to look at the biological receptor of insulin as a basis for assay, and necessitated the search for suitable insulin receptors that mediate the physiological action of insulin. The source of receptor chosen for the radioreceptor assay was the IM9 cultured human lymphocyte cell line first suggested for assay use by Gavin and co-workers in 1975 [20] and developed in Wellcome laboratories by Baxter [21].

Thus, through the use of human cells, the "potency" of insulin for human use can be tested in a human *in vitro* system. In the assay, insulin binding is detected by using a competition between unknown or reference insulin and insulin labelled with ^{125}I. The amount of radioactivity bound to cells is the measured parameter and, as in the radioimmunoassay, the higher the insulin concentration in the test material, the lower the binding of ^{125}I.

The radioreceptor method was used in 1983/1984 to assay samples of human, porcine and bovine insulin provided by the National Institute for Biological Standards and Control, London, as part of a World Health Organisation Collaborative Trial. Table 1 shows the radioreceptor results compared with those obtained from the same samples using the mouse blood glucose method. Potencies for the bovine insulin samples correlated well in the two methods, but the radioreceptor technique provided estimates for the pork and human insulin which were about 25% higher than those obtained by the animal method.

Although these results may have appeared anomalous, they could indicate a genuine intrinsic higher binding avidity in man of pork, or human insulin compared with beef. This is not incompatible with their respective chemical structures, in which respect porcine insulin is more closely related to human insulin than is bovine insulin. Asplin, Hartog and Goldie [22] reported an average 22% reduction in daily insulin requirement in a group of 58 diabetics transferred from beef insulin to purified porcine insulin; this reduction in dose matches well the differences in potency observed using the radioreceptor and mouse blood glucose methods.

Table 1
Analysis of insulin samples in a World Health Organisation collaborative trial using the mouse blood glucose (MGB) method and radioreceptor (RR) assay

Type of insulin	Sample	Mouse blood glucose potency u/mg	Radioreceptor potency u/mg	Ratio RR/MGB	
Beef	A	25.7	26.6	1.04	1.00
	D	27.9	26.5	0.95	
Pork	B	25.2	32.1	1.27	1.28
	E	25.3	32.7	1.29	
Human	C	25.9	31.0	1.20	
	F	25.8	33.7	1.31	1.32
	83/567	26.0	37.7	1.45	

Additional tests

In addition to the determination of potency, two further tests using animals on insulin formulations appear in the 1980 edition of the British Pharmacopoeia — these are Prolongation of Insulin Effect and Insulin in Solution.

With regard to the test for Prolongation of Effect of insulin, soon after the discovery of insulin in the 1920s the early crude insulin formulations demonstrated a slow onset of response and a slow recovery. As insulin purification techniques began to improve and non-insulin proteins began to be eliminated from the early insulin formulations, it was noted that the insulin response became sharper with a more rapid onset and a faster recovery. It then became necessary, in some circumstances, to give diabetics more than one injection of insulin a day in order to hold the blood glucose levels down over the 24 h period. Consequently a "delayed action factor" was built into certain formulations. This was achieved by complexing the insulin with a protein such as protamine sulphate or more recently by precipitating the insulin, complexed with zinc, as crystals which when injected take some time to dissolve into the blood stream of the patient.

Initially, rabbits were used to test insulin formulations for prolongation of insulin effect, but Stewart and Smart [23] at Wellcome showed in 1953 that the guinea pig was more sensitive to qualitative differences in insulin preparations than was the rabbit. The speed of dissolution or breakdown of an insulin complex is related to its bioactivity in the body. Therefore insulin preparations may be characterised by a physical method. Graham and Pomeroy [24] showed that an *in vitro* dissolution test could reflect the type of prolongation in animals, a technique which we were able to copy and which produced some excellent results (Fig. 4).

However, after examining the results of the *in vivo* tests obtained over the years, manufacturers and pharmacopoeial authorities realised that no batch of insulin had failed to reach the market simply on the basis of inadequate prolongation of effect. This test is, therefore, considered to be superfluous for routine quality control, provided that the physico-chemical characteristics of the insulin complex can be clearly defined. The Prolongation of Action test has now been removed from the current British Pharmacopoeia.

The test for Insulin in Solution which has been linked to the biological assay is a measure of the amount of activity contained in the supernatant derived from a

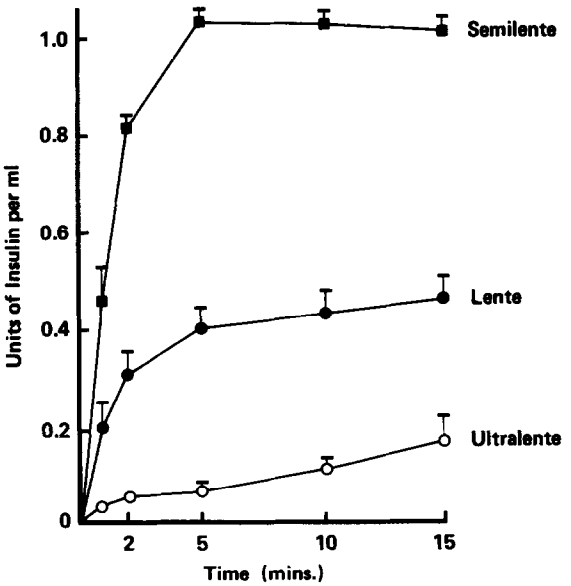

Figure 4
Dissolution profiles of insulin zinc suspension formulations.

preparation of a complexed insulin (e.g. Insulin Zinc Suspension). It has not been difficult to convert the biological test for Insulin in Solution to a physico-chemical test with the reservation that the latter is sufficiently sensitive to measure the very small amounts of insulin that might be in the solution. HPLC has provided a very satisfactory method, although for this purpose, even radioimmunoassay could have been used.

Future trends

So, what of the future? Two improvements in technology have, over the last few years, opened the door to a new era of insulin testing. Firstly, improvements in manufacturing and purification processes have resulted in the production of very pure, virtually single-component insulins. This was followed in 1986 by the establishment of separate international standards for human, bovine and porcine insulins by the World Health Organisation. Secondly, the advent of HPLC columns with greater resolving power has enabled further development by workers in this field, including Fisher and Smith [25] of Wellcome, of precise chromatographic assay of insulins. This enables species to be identified and the proteins in the formulations can be separated and quantified. The procedure involves the introduction of insulin formulations to a C3 Ultrapore column and the reading of the peaks produced at 214 nm. Using this technique, peaks of insulin and desamido insulin can be identified and quantified. It is the insulin and desamido insulin components which contribute to the biological activity, and calculation of the areas under the peaks produced by these can provide a quantitative analysis of the insulin and insulin-like components present in the formulated injections. However, bulk crystalline insulin should still be assayed by an *in vivo* method to ensure the presence of bio-activity.

Table 2
A comparison of the mouse blood glucose and HPLC methods in determining the potency of insulin formulations

Sample	Potency u/ml Mouse blood glucose method		HPLC method*	
Neutral soluble insulin				
1	101.3	99.8	96.0	97.4
2	100.6		98.7	
3	95.3		93.9	
4	101.8		101.0	
Isophane insulin				
1	99.7	101.9	95.5	101.1
2	106.5		99.1	
3	101.8		105.3	
4	99.6		104.5	
Insulin zinc suspension				
1	105.3	102.6	97.5	94.4
2	96.5		96.7	
3	105.2		91.3	
4	103.3		92.0	

*Calculated assuming 26 u/mg of bulk crystalline insulin.

Unfortunately, desamidation of insulin can increase after the preparation of a formulation and can lead to the presence of mono-, di- and poly-desamido insulins depending on the pH of solution and temperature of storage. With satisfactory formulations and under satisfactory storage conditions, little other than mono-desamido insulin is formed and its slightly reduced biological potency can be accommodated in the HPLC assay calculation. Table 2 shows a comparison between the HPLC results and those produced by the mouse blood glucose method when assaying insulin formulations in an early study.

With the co-operation of the principal pharmaceutical companies, the European Pharmacopoeia Commission have organised a collaborative trial at the end of 1988, so that candidate EP Biological Reference Insulin Preparations can be calibrated against the appropriate international standard by both biological assay and HPLC.

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