REVIEW ARTICLE

RECENT ADVANCES IN BIOLOGICAL STANDARDISATION

WITH PARTICULAR REFERENCE TO THE ASSAYS OF DIGITALIS AND POSTERIOR LOBE PITUITARY EXTRACTS

BY G. F. SOMERS, Ph.D., B.Sc., Ph.C.

Lecturer in Pharmacology, The School of Pharmacy, University of London

GENERAL PRINCIPLES

THE most notable advances in the biological standardisation of drugs, hormones and therapeutic substances during the past 20 years have been in the development of experimental procedures and assay techniques suitable for statistical analysis. This has been largely due to the recognition of the growing importance of statistics and to a close collaboration between the assayist and the statistician. The development of bioassays on fundamentally sound principles began with the establishment of a standard reference preparation by Ehrlich as long ago as 1897. However, laboratory workers were slow in adopting this principle and for many years afterwards potency continued to be defined in animal units. Such units were extremely variable and agreement between different laboratories was impossible. Due to the pioneer work of Dale, Hartley, Burn, Gaddum and Trevan bioassays were gradually established on scientifically sound foundations and biological standards adopted on an international basis. Thus a uniformity in the potency of therapeutic substances was ensured throughout the world and, e.g., a diabetic person receiving insulin in the western hemisphere can be assured of receiving the correct dosage in, say, Bombay. Reviews on the establishment, properties and uses of international standards have been published by Dale,[1] by Hartley[2] and by Miles.[3,4]

Much of the progress in biological standardisation has been due to a recognition of the principles of experimental design and a realisation of the need for a strict statistical control. Thus in the 2nd edition of "Biological Standardisation" by Burn *et al.*[5] statistical methods have taken pride of place. This strongly underlines the function of the statistician not only in analysing and assessing the precision of the data obtained but also in the planning of the experimental procedures prior to the assays themselves. Among important contributors to the development of statistical methods are Bliss, De Beer, Knudson and Miller in the United States and Burn, Fieller, Finney, Gaddum, Gridgeman, Irwin, Trevan and Wood in this country. A recent review on the statistical foundation of biological assays has been published by Finney,[6] and books by Emmens,[7,8] Burn *et al.*,[5] Coward[9] and Finney[10] describe the use of statistical methods in bioassay.

The general principles to which any biological standardisation must conform have been defined by Dale,[1] and further characterised by

Gaddum,[11] and by Bliss.[12] These have been summarised by Emmens[13] as follows:—

(1) A standard reference preparation must be used simultaneously with the preparation under test.

(2) The assay must provide a valid, unbiased estimate of the potency of the preparation under test and of the limits of error of this estimate at any required probability.

(3) The assay must provide evidence that the actions of the preparation under test and of the standard preparation do not differ.

(4) The most accurate test method will be that for which the quantity s/b is minimal; where s is the standard deviation of an individual result and b is the slope of the dose-response line.

(5) The living material receiving each dose of the standard and unknown must be as uniformly distributed among such dosage groups as is possible. Potential sources of variation such as differences in response between litter-mates, sexes or strains of animal, must be so allocated to dosage groups that their influence can be isolated and examined in the subsequent statistical analysis.

The Choice of an Assay Method

The choice of an assay method is largely influenced by factors such as the time, cost, materials and labour involved. These are of primary importance in the commercial production of drugs and therapeutic substances. Thus the rabbit assay of insulin is to-day largely obsolete and the mouse method is preferred because of its rapidity and simplicity. The method used should be specific, but when the activity of the standard and test preparations are due to identical active principles, as they should be, it is immaterial whether or no the response measured is the same as the therapeutic effect for which the preparation is to be used. This is not so when the active principles differ or are present as heterogenous mixtures as in digitalis. In such cases the assay method must be carefully defined or results obtained in different laboratories will be conflicting.

The Design of Experiments

In the planning of new experimental techniques a biometrician should be consulted before embarking on the experiments themselves. Good planning can save much tedious arithmetic and considerably improve the precision of the results obtained. The design of the experiment will largely depend on the type of response measured and whether or not the experiment can be repeated on the same test object. This is not always possible. In toxicity tests, which are based on a quantal response, the reaction which is an "all or none effect" can be observed only once, and more complicated and time consuming statistical procedures are necessary for the evaluation[10] of such tests. In assays based on a quantitative response it is often possible to repeat the doses many times on the same test object. By suitable arrangement of the standard and test preparations sources of error such as variation between animals, days and positions

can be eradicated from the final error of the estimate which is dependent on the residual variance. A valuable design is randomisation according to published tables (Latin squares).[14] This has been adopted in the assay of insulin,[15] histamine,[16] adrenaline,[17] curare[18] and posterior lobe pituitary extracts.[19] In some cases it may be difficult to eradicate from the experimental comparisons the effect of changing sensitivity and this may only be partly achieved by the random dose method. Here the experimental design of Vos[20] is preferred, the dose of the standard preparation remaining constant and being alternated with 3 ascending doses of the test preparation. This design is described in the United States Pharmacopeia XIV[21] for the assay of adrenaline and posterior lobe pituitary extracts. It does not give any information on the regression of the standard preparation, however. Other experimental designs include cross-over tests[22] and twin cross-over tests.[23,24,25] Where experimental methods do not permit of statistical evaluation it is often necessary to develop new methods, as in the assay of posterior lobe pituitary extracts. Types of experimental designs used in biological assays have been reviewed by Finney[6,26] and by Emmens,[7,8] and all biological workers will be familiar with the book by Fisher[27] on the design of experiments.

The Assay of Digitalis

The assay of digitalis has long presented the pharmacologist with a problem, and a vast amount of time and energy has been devoted to the investigation of methods which might give a true indication of its activity in man. However, digitalis leaf and galenical preparations consist of a variable mixture of active glycosides and any biological assay will therefore be fundamentally unsound since it will not comply with the first principles of biological standardisation. It is not surprising therefore that results obtained in different species of animals—frogs, cats, guinea-pigs and pigeons—may be conflicting and discrepancies observed with the therapeutic effect in man. Biological standardisation cannot avoid the sources of error associated with impure preparations. Methods should be closely defined and if possible bear a close relationship to the therapeutic effect in man if any reliance is to be placed upon them at all. A major discrepancy in the assay of digitalis lies in the route of administration. In the cat, guinea-pig and pigeon assays toxicities of the test and standard samples are compared by the intravenous route while in man digitalis is administered orally. By mouth even the pure glycosides are not completely absorbed and degradation occurs in the alimentary tract. The effect measured in animals, toxicity, differs from the therapeutic effect in man. While toxicity and therapeutic effectiveness appear to be related, even this is not certain.[28] Attempts have been made to standardise digitalis in man by Gold *et al.*[29] They used two reactions; slowing of the heart and an inversion of the T-wave in the electrocardiogram. In one series, using slowing of the heart rate as their response, the result of a comparison of the standard powder with an unknown in man agreed fairly well with those of the cat assay, while agreement between the human assay and the frog assay was not so close. In another series, however,

using the T-wave test there were marked discrepancies between the human and cat assays.

Therapeutically exact dosage in prolonged treatment with cardiac glycosides is important. This can only be assured by the use of chemically pure crystalline glycosides, the dosage of which can be accurately prescribed. However, while physicians continue to show a preference for the mixed glycosides biological standardisation will remain necessary.

Methods of Assay.

The international standard for digitalis is a sample of dried leaf. It was recently necessary to replace the second standard, which was nearly exhausted, with a new third international standard. One unit is contained in 0·076 g. of this new standard compared with 0·08 g. of the second standard. In establishing the third standard a series of collaborative assays was undertaken in 16 laboratories in different countries. The results and analysis of the different assays have been published by Miles and Perry.[29]

The chief assay methods for digitalis use respectively the frog, guinea-pig, cat and pigeon. Whether or no the assay of digitalis should be restricted to any one method is debatable, but if several alternative procedures are in use it is inevitable that using an heterogenous standard results obtained in different laboratories will be conflicting. Theoretically it would appear desirable that each sample be assayed on several species of animals, as was done in the establishment of the third International Standard,[30] and to take the weighted mean which might be expected to give a result most comparable with man. However, this expensive and time consuming procedure would be impossible on a routine basis. It may be argued that in the collaborative assay in the establishment of the third International Standard agreement between different laboratories and methods was good, but both the second and third standards represented pooled material from different sources and the constitution of the glycosides in the two samples was probably more similar than usual.

The cat method, originally introduced by Hatcher and Brody[31] and perfected by Lind van Wijngaarden,[32] is considered the most satisfactory assay method since it gives almost identical results in the various laboratories,[33] and the results are in general agreement with assays in humans.[29] However, it is difficult to obtain cats in sufficient numbers and in this country the guinea-pig method of Knaffl-Lenz[34] is most widely used. This method has not been popular in the United States, probably because of a lack of agreement with the cat assay.[35] More recently American workers have adopted an intravenous pigeon method first described by Haag and Woodley,[36] who showed that the results obtained agreed favourably with those of the cat method. While the frog method was popular at one time, it has now become largely obsolete in this country and the United States, results obtained in warm blooded animals, which are closer related to man, being preferred. Gold and Cattell[37] reported that frog and man reacted differently to digitalis and the results might be misleading.

In spite of the shortcomings of digitalis assay methods, they should be designed to give the greatest possible precision and the methods should be closely defined if comparative results are to be obtained in different laboratories. It is on these grounds that the methods at present official in the British Pharmacopœia[38] can be criticised. The first frog method described is based on a standard dose response curve originally established by Trevan.[39] It has been shown by Miller[40] and by Miles and Perry[30] that the slope does not remain stable, even in one laboratory, for any length of time, and that the slopes obtained in different laboratories are not homogenous. Admittedly the British Pharmacopœia does suggest a test for slope, but this is impossible with single dosage groups. The alternative frog method is the correct one, the assay being conducted by determining the overnight mortalities in 4 groups of frogs. This is based on a sound statistical foundation enabling the slopes of the standard and test to be compared as an integral part of the assay and the error easily calculated. The method is more adequately described by Miles and Perry.[30]

Both the official cat and guinea-pig methods, in which the diluted tincture is slowly and continually infused intravenously into the anæsthetised animal until the heart is arrested, can also be criticised. In neither case is it necessary to make a simultaneous comparison of the standard and test preparations, the assays being based on the establishment of a standard laboratory figure which is required to be redetermined at a rather indefinite period "from time to time." Bliss[33] has shown that the sensitivity of cats in any one laboratory would only remain constant over a period of 15 days. Similarly in guinea-pigs Jacobsen and Larsen[41] found variations in the sensitivity of the animals to occur and adopted the procedure of always comparing the standard and test samples alongside each other. If such a procedure is adopted it is not logical to use unequal groups, 14 animals on the standard and 6 on the test. Equal numbers should be used in each group and the number determined by the required limits of error, as is done in the U.S. Pharmacopeia.

The British Pharmacopœia assumes that in both cats and guinea-pigs the toxicity of digitalis is related to body-weight, but this is open to question.[5] Heavier cats are relatively more sensitive than lighter ones on a body-weight basis,[30] especially when the continuous infusion method is used. Miles and Perry[30] suggest that this is due to the heavier cats not receiving a lethal dose of a rapidly acting toxic glycoside at as early a stage of the infusion as the lighter cats and they will therefore be exposed for a longer period to the effect of the slowly-acting glycosides and the time of death may thus be relatively earlier than in the lighter cats. It is for this reason that the U.S. Pharmacopœia XIII adopted a procedure of intermittent injections, suggested by Bliss,[33] the injections being made in fixed doses at 5-minute intervals until the heart was arrested. This method allows the slower acting glycosides time for fixation by the tissues. The time for death is standardised by requiring that the average number of doses for any given dilution to produce death should be not less than 13 and not more than 19. The standard and test were required to be tested alongside each other and the assay completed within 15 days.

Similarly in guinea-pigs Miles and Perry[30] showed that the coefficients of correlation between the logarithms of the lethal doses and the body-weights were highly significant and that the heavier were more sensitive than the lighter ones. Miles and Perry emphasise that there is a danger in the use of this method for routine assays the results being biased unless the weights of the guinea-pigs in the two groups are carefully controlled.

The method now official in the United States is the pigeon method of Haag and Woodley[36] as modified by Braun and Lusky.[42] The pigeon is readily obtainable and cheap and the results obtained more consistent than with the cat. It is claimed that only 6 to 8 birds are required in the standard and test groups for a standard error of ±5·7 per cent. Since this method is at present little known in this country it will be described in detail. It should not be confused with the method of Hanzlik[43] which is based on the emetic action of digitalis in pigeons. Adult pigeons are selected for the test so that the weight of the heaviest does not exceed twice the weight of the lightest. They are randomly distributed into groups as nearly alike as possible with respect to breed, sex and weight, so that the average weights of the two groups do not differ by more than 30 per cent. For the assay the pigeons are lightly anæsthetised with ether and an alar vein exposed and cannulated. The diluted tinctures are injected from a small bore burette and made at 5-minute intervals by quickly infusing a volume of the diluted tincture equivalent to 1 ml./kg. of body-weight, until the pigeon dies from cardiac arrest. The test and standard tinctures are previously diluted in such a way that the fatal dose will be diluted to 15 ml. with isotonic saline solution. The requirement is made that the average number of doses required to produce death is not less than 13 or greater than 19. A total of not less than 6 pigeons is used for the standard preparation and 6 for the test, the number being increased if the standard error exceeds 0·08 unit. This method has much to commend it, the technique is simple, animals easily obtained and the end-point —dyspnœa and terminal convulsions—extremely sharp.

Posterior Lobe Pituitary Extract

Posterior lobe pituitary extract, like digitalis, consists of a mixture of active principles. Three of these are used clinically—the oxytocic, vasopressor and antidiuretic hormones. Only two, the oxytocic and vasopressor activities have been almost completely separated. Liquid Extract of Pituitary (Posterior Lobe) was required in the British Pharmacopœia 1932 to contain 10 I.U./ml. and to yield qualitative tests for vasopressor and antidiuretic action. The British Pharmacopœia 1948 contains three preparations:—(*a*) an injection of pituitary standardised to contain 10 I.U. (oxytocic) /ml. and required to be assayed for antidiuretic or pressor activity only if these are stated on the label; (*b*) an injection of oxytocin standardised to contain 10 I.U. (oxytocic) /ml. and not more than 0·5 I.U. (pressor) per ml.; (*c*) an injection of vasopressin standardised to contain 10 I.U. (pressor) per ml.

Standard.

The present international standard is a sample of dried pituitary powder established in 1940 when stocks of the previous standard, established in 1925, were becoming exhausted. Fortunately the new standard had practically the same activity and composition as the previous one, hence it was unnecessary to redefine the unit as was required with digitalis. The unit is defined as the activity of 0·5 mg. of this material for oxytocic, pressor and antidiuretic assays.

Methods of Estimation.

The methods of assay of pituitary (posterior lobe) extracts have been reviewed by Thorp[44] and by Stewart.[45,46]

Oxytocic activity. The method described in the present British Pharmacopœia is the same as that in the B.P. 1932, the assay being performed on the isolated uterus of the virgin guinea-pig. This method was introduced by Dale and Laidlaw[47] and has long presented practical difficulties; suitable uteri are difficult to obtain, many showing an inherent rhythm and a poor differentiation between graded doses of the same extract. The method is based on a scheme of matching doses and is unsuitable for statistical analysis, the experimental error being indeterminable from individual assays but only from separate experiments. Various workers have improved the method.[48,49,50] Some have attempted to improve the assay by modifying the concentrations of magnesium and calcium ions in the Ringer solution,[51,52] thus increasing the sensitivity and preventing spontaneous alternating rhythms. However, there is evidence that vasopressin exerts a considerable degree of oxytocic activity in the presence of magnesium. Attempts to design the guinea-pig uterus method for statistical evaluation have not been very satisfactory due to the difficulty in obtaining a large number of repeatable responses. The virgin guinea-pig method is therefore far from satisfactory and has been largely superseded by the rat uterus method[55,19] and the chicken blood pressure method.[56,57,58]

The rat uterus method employs the isolated uterus of the non-pregnant diœstrous rat. Suitable uteri are easily obtained and since the doses can be repeated at 3- to 4-minute intervals the 4-point assay described by Schild[16] and by Holton[19] can be used, thus enabling the experimental error to be determined for each experiment. An assay can be completed within 4 hours and limits of error ($P = 0{\cdot}95$) within ± 20 per cent. easily obtained.

The chicken depressor method is official in the United States Pharmacopeia XIV and is based on the depressor action of vasopressin on the blood pressure of the cockerel. Blackwell Smith and Vos[58] describe a 4-point assay but changes in sensitivity during the dose schedules increases the error of the assay and, to overcome this, Thompson[59] used the experimental design described by Vos.[20] If the standard and test materials are similar to each other the experimental design of Vos gives the more accurate results, but should the standard and unknown materials be dissimilar then the Schild[16] experimental design should be used when an

analysis of variance is possible and one can determine whether the assay is valid or not.

Pressor activity. The official method for the assay of pressor activity is on the blood pressure of the spinal cat.[47,60] Since it is necessary to allow intervals of one hour between doses a statistical design is impossible, and the potency of the test preparation can only be assessed by bracketing it between doses of the standard extract. With the anæsthetised dog[61] doses can be given at 15-minute intervals and while the discrimination between doses is not as good as with the cat the larger number of doses which can be given results in as great an accuracy.[46]

The anæsthetised rat has been proved a satisfactory preparation for the determination of pressor activity and is extremely sensitive.[62,63,64] The assay is not interfered with by traces of histamine in commercial powders. Landgrebe *et al.*[64] used anæsthetised male rats, the central nervous system of which was pithed caudally from the anterior tip of the pelvic girdle to eliminate fluctuations of the blood pressure. Injections were made into the femoral vein at 15-minute intervals and the blood pressure recorded from the carotid artery. Landgrebe *et al.* used the method of "matching doses" but a randomised block design[16] could be used.

Antidiuretic activity. While numerous methods have been described for the determination of antidiuretic activity few have used modern statistical methods in designing their procedure or in evaluating their results. The vasopressor hormone and antidiuretic hormone are believed to be due to the same active principle,[65] but Heller[66,67] has prepared from the vasopressor fraction an extract containing a high proportion of anti-diuretic activity and very little vasopressor activity.

The method most widely used for the determination of the antidiuretic hormone is due to Burn[68] and this is the one described in the British Pharmacopœia. The assay depends on the time for the maximum rate of urine excretion in rats following administration of water by stomach tube and posterior lobe pituitary extract by injection. 8 rats are used on the standard preparation and 8 on the test in a cross-over technique, the test being repeated 2 or 3 days later when the rats which received the standard now receive the test preparation and *vice versa.* The urine excreted is measured at 15-minute intervals from which the time for maximum excretion is determined. The potency is calculated by reference to a standard dose-response curve which should be predetermined for each laboratory and breed of rats. The error of the assay cannot be assessed from a single assay and the Pharmacopœia states that "the data at present available do not permit of a sufficiently accurate determination of the limits of error."

Numerous modifications of Burn's method have been described. Gilman and Goodman[69] obtained a more consistent response by giving a preliminary hydrating dose of water three hours before the commencement of the test, while Silvette[70,71] gave a single dose of 0·2 per cent. saline solution by intraperitoneal injection and measured the total volume of urine excreted during a period of 6 hours. Krieger and Kilvington[72] measured the volumes of urine excreted at intervals of 15 minutes over a period of 6 hours and measured the area of a plotted curve with a

planimeter. Ham and Landis[73] have closely examined the various factors in an antidiuretic assay and conclude that the estimation of chloride excretion is preferable to measurements of the urine volume.

Ginsburg[74] has recently described a method for an assay using rats, which can be readily analysed statistically. This method is based on a regimen of water administration described by Birnie *et al.*[75] Groups of rats are given 2 doses of water by stomach tube with an interval of one hour between doses, followed by a third dose one hour later when the injections of pituitary extract are given. The urine is collected from the rats placed in individual metabolism cages and measured at 60, 90 and 120 minutes from the time of the injections. A 4-point assay procedure is described from which the potency and its error can be determined by standard statistical procedure. A single assay can be completed in 5 hours.

Other methods of determining antidiuretic activity have been described in mice,[76,77] dogs[65,78,79] and rabbits.[80,81,82]

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