MICROBIOLOGICAL ASSAY

An Introduction to Quantitative Principles and Evaluation

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PREFACE

During the past decade there has been world-wide increased awareness of the importance of quality control of pharmaceutical preparations. The World Health Organization has at the same time been active in encouraging and assisting member states to strengthen their national quality control systems. As a result, many nations are planning either new regulatory laboratories or the extension of existing facilities.

The increased importance of microbiological assay of antibiotics is indicated by the existence today of about forty International Biological Standards and Reference Preparations as compared with one in 1948.

Quality control of this large and important group of modern medicines is a major task of pharmaceutical analysts both in industry and the laboratories of government regulatory authorities. Although physical and chemical methods of examination may in some cases suffice, in many cases microbiological assay remains the only method of assessment of potency. The value of microbiological assay for the estimation of certain vitamins and amino acids is also well established.

The method has the advantage that it *can* be carried out without highly specialized and expensive equipment. There have been developments in mechanization and automation of individual steps in assay procedures. Completely automated systems are also available. These call for relatively large capital expenditure which can be justified only when the examination of a sufficiently large number of samples is envisaged. It seems likely that manual methods will continue to be widely used for many years.

As the same basic procedures are applicable to a wide range of antibiotics, microbiological assay is well suited for the routine examination of large numbers of samples by well-trained and well-supervised personnel. The full potential of the method is achieved in those laboratories in which it is treated as a branch of quantitative pharmaceutical analysis and applied with an awareness of the chemical, physical, and mathematical as well as biological principles involved.

Often, however, the method is applied empirically with inappropriate and inefficient designs, poor reproducibility being attributed to "biological error."

While random variation of individual responses is a significant feature of most microbiological assays, there seems to be no reason to suppose that this is biological in origin. The concept of biological variation is based on the differing responses of individual subjects to the same stimulus. In *macro*biological assays where the number of test subjects receiving the same stimulus is small, random variation of mean responses is to be expected. In *micro*biological assay, the situation is quite different. Inocula are in most cases measureable in terms of millions of organisms so that mean responses to the same stimulus under the same test conditions are likely to be very similar. The problem facing the analyst is not uncontrollable biological variation of the organism but how to regulate the physical conditions of the test so as to ensure that the effect of the stimulus is not modified by unwanted influences.

Despite such control of physical conditions, as in any assay method, random variation of responses remains and may be measured by statistical techniques.

In routine *micro*biological assay it is in the author's opinion neither necessary nor economically justifiable to carry out a statistical evaluation leading to confidence limits for each individual assay. Moreover, routine calculation of such limits can be a trap for the unwary, giving false confidence in estimated potencies when bias due to poor techniques may be substantial yet overlooked.

It is for these reasons that statistical evaluation of assays is treated in Chapters 6 and 7 quite separately from the potency calculations of Chapters 2–4.

The need to be aware of the principles of statistical evaluation becomes apparent in Chapter 8 in which features of assay design such as replication, number of dose levels, and spacing of dose levels are discussed.

Many supervisors of microbiological assay laboratories are by inclination bacteriologists rather than analysts; thus, the mathematics has been kept as simple as possible. It is assumed only that the reader has an elementary knowledge of algebra and has been introduced to the basic concepts of statistics. Although some calculations are lengthy, the individual steps are nothing more than simple arithmetic. Practice in the application of these methods helps the beginner gain a better understanding of their principles. A word of warning: statistical evaluation as normally applied to simple individual assays gives an idea of the capability of the method, or to put it another way, it gives an idea of the limitations imposed by technique and assay design. It does not take into account the gross errors or biases which can and do arise from neglect of the special features of the assay method and the principles of quantitative analysis. Thus, evaluation of *precision* is no substitute for painstaking efforts to control physical conditions and operating procedures so as to obtain an *accurate* estimate of potency.

The examples given in this work have been collected from several laboratories in North America, Europe, and Asia over a period of more than fifteen years. Many represent routine work of these laboratories and do not necessarily indicate the author's ideas on either analytical technique or assay design. The examples have been chosen using the criterion that they illustrate a representative selection of designs available to the microbiological analyst.

The use of poor assay design is widespread. Such designs have been included here very deliberately so as to draw attention to their disadvantages.

This volume has developed from notes written in Turkey in 1968 with the limited aim of explaining the mysteries of the calculation procedures employed in microbiological assay. Although this remains an important aspect of the work, there is now stress on assay design, the elementary principles on which the various methods are based, as well as general principles of pharmaceutical analysis.

No attempt is made to give detailed descriptions of individual assay methods. This information is available from sources such as the international and various national pharmacopeias, the United States Code of Federal Regulations, Kavanagh's "Analytical Microbiology" Volumes I and II, Barton-Wright's "Microbiological Assay of the Vitamin-B Complex and Amino Acids," and György's "Vitamin Methods" Volumes I and II.

More advanced accounts of principles of microbiological assay are to be found in certain chapters of Kavanagh's "Analytical Microbiology" Volumes I and II. For advanced accounts of assay design and evaluation the reader may consult Finney's "Statistical Method in Biological Assay" or Bliss's contribution to György's "Vitamin Methods" Volume II.

It is hoped that this work will succeed in its aim of providing an elementary introduction to the principles of microbiological assay, assay design, and calculation procedures and so help to encourage a less empirical approach to the subject.

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It is natural that the end product does not necessarily reflect accurately the views of everyone whose help is acknowledged and that any shortcomings are of course my own responsibility.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Philosophy of Biological Assay

The need for standardization of products affecting our lives in literally thousands of ways is a well-established fact. It was expressed picturesquely in advertisements for a certain brand of shaving soap, which was claimed to produce just the right amount of lather: "Not too little, not too much, but just right!"

The need for standardization assumes much greater importance where medicinal substances are concerned. In some cases the margin between too little (an ineffective dose), and too much (a toxic dose) may be relatively small. In other cases, while an unnecessarily high dose may not be toxic it could be undesirable on economic grounds.

Many medicinal agents consist of a single active substance that can be characterized completely in terms of its chemical, physicochemical and purely physical properties. A specification may be devised for a pharmaceutical grade taking into consideration the properties of the pure substance and making allowance for tolerable levels of impurities. Certain impurities that are expected to arise from the manufacturing process may be limited by specific tests.

Other medicinal agents, however, particularly those of natural origin, may be of more variable character. They may consist of a mixture of chemically related substances differing quantitatively and qualitatively in their biological effects. They may also include chemically unrelated substances that have biological activity. The activities of the different components may be either mutually compatible or antagonistic. They may even be synergistic.

Not uncommonly, a substance becomes of recognized therapeutic value before its exact chemical composition has been ascertained.

Such problems existed long before the discovery and commercial production of antibiotics with which we are largely concerned in this present work. Well-known examples include the alkaloids of ergot, the solanaceous alkaloids, digitalis glycosides, and the purgative drugs containing anthraquinones and related substances.

When for any reason a potentially valuable medicinal agent cannot be defined in terms of its chemical or physicochemical properties, then the obvious alternative is to consider its biological properties.

Unfortunately, biological properties cannot be simply quantified. Attempts to measure potencies by purely biological means have never been successful

due to the inherent variability of the biological system. It is true that some tests such as those described for pyrogens and toxicity in certain pharmacopeias are defined in terms of the effect of the drug on a group of animals under specified conditions. However, these are limit tests. Moreover, it is likely that results would not be closely reproducible in different laboratories or even in the same laboratory on different occasions. The limitations of these methods are recognized and accepted in the absence of better alternatives.

For truly quantitative work the problem of variable response of the test organism is overcome by the use of comparative methods. A quantity of active substance is set aside and designated the standard preparation. The effect of any sample on a biological system can be compared with this standard preparation to obtain a quantitative relative potency.

The method used for comparison of the two preparations may be macrobiological, such as the assay of insulin using mice, or microbiological, such as the assay of streptomycin or tetracycline.

1.2 Basic Techniques and Principles

The two most commonly used methods of microbiological assay will be referred to as the plate (or agar diffusion) method and the tube method. The basis of both these methods is the quantitative comparison of the effect of two substances on the growth of a suitable microorganism in a nutrient medium. The two substances are a standard and a sample whose potency is to be determined. The effect may be to inhibit growth, as in the case of antibiotics, or to promote growth, as in the case of vitamins and amino acids.

The practical procedures for both these methods are illustrated here only in outline by two typical simple antibiotic assays.

(a) *The plate assay of penicillin.* Nutrient agar is melted and its temperature reduced to 48°C. A small volume of a suspension of a penicillin-sensitive microorganism (e.g., *Staphylococcus aureus*) is added by pipet and gently but well mixed to give a uniform dispersion in the agar medium. A suitable volume (about 15–20 ml) of this seeded agar is pipetted into a petri dish to give a layer of uniform thickness (about 3–5 mm).

After solidification of the seeded agar the plate is ready for use and may be refrigerated until required.

Two or more concentrations of penicillin solutions prepared from both reference standard and test sample are applied to reservoirs at appropriately spaced positions on the plate. These positions may be in accordance with different randomized patterns for each plate in a set comprising one assay.

One experimental design, however, uses only a single pattern for all plates (see Example 8).

Suitable patterns for use with petri dishes are given in Appendix 1. Various forms of reservoirs are in use:

(1) Small cylinders of the agar are cut and removed using a cork borer, or better, a specially made 8-mm diam stainless steel punch is convenient.

(2) Specially designed sterilized stainless steel cylinders are placed on the surface of the agar.

(3) Small sterilized electrical ceramic insulators (fish spine beads) are used. The beads are dipped into the test solution, surplus liquid is drained off, and then the bead is placed on the agar surface.

(4) Small filter paper disks are used in a similar manner to the beads.

For the first two of these procedures a standard volume of test solution is added to each reservoir. This may be measured simply as a constant number of drops added from a standard dropper, or the reservoirs may be filled almost to the brim, or a semiautomatic pipette may be used.

The solution is allowed to diffuse into the agar at room temperature or lower for an hour or perhaps more, and then the plates are incubated, usually overnight.

After incubation, clear zones surround the point of application of the antibiotic, whereas in other parts of the plate growth of the microorganism causes turbidity.

Zone boundaries are usually clearly defined, although the sharpness of definition varies according to test organism, the density of the inoculum, the antibiotic, etc.

Inhibition zone diameters are measured. The relationship between mean responses (zone diameters) to each test solution and the concentration of that test solution is the quantitative basis of the assay.

(b) *The tube assay of neomycin.* A series of concentrations of neomycin standard solutions are prepared, as well as one or more solutions of the sample within the same concentration range as the standard. For one series of tubes, 1 ml of each solution is added to a separate test tube. This is followed by 9 ml of a nutrient medium inoculated with a suspension of the neomycin-sensitive test organism *Klebsiella pneumoniae.* Usually two or more series of tubes are included in each assay. The tubes are incubated for about 4 hours; then the growth is stopped in all tubes at the same time (immersion in a water bath at 80°C is very satisfactory—higher temperatures may result in coagulation of protein). The growth of the organism is estimated by the turbidity measured in a suitable photometer.

The mean inhibition of growth corresponding to each test solution in the set is the basis of calculation of potencies. Lower turbidities correspond to higher concentrations of antibiotic.

Despite their widely differing techniques, these two methods have a common basis in that they depend on the following principles:

(1) Comparison of a sample of unknown potency with a standard substance of known defined activity.
(2) Measurement of the inhibiting effect on the multiplication of the test organism.
(3) The existence of some form of quantitative relationship between concentration of active substance and response.
(4) This quantitative relationship is the same for the sample as for the standard.

The forms of these relationships and convenient ways of calculating potencies are described in detail in Chapter 2 for agar diffusion assays and Chapters 3 and 4 for tube assays.

Both plate and tube assays of growth-promoting substances (g.p.s's) such as vitamins and amino acids differ from assays of growth-inhibiting substances in that the response is opposite. In plate assays the point of application of the test solution is surrounded by a turbid zone of exhibition contrasting with its relatively clear surroundings. In the tube assay increasing doses of test solution cause increasing growth of the organism.

Both techniques have the following requirements:

(1) The test organism must be dependent for growth on the presence of the substance to be assayed.
(2) Addition of graded doses of the substance to be assayed (both sample and standard) should result in graded responses on incubation.
(3) The nutrient medium for the test must contain an excess of all substances required by the test organism except the substance to be assayed. This substance should be absent from the basic medium.
(4) Apart from the substance to be determined, no other substance that may be present in the sample should be capable of promoting growth of the test organism or of modifying its growth. This is a factor to be considered in choosing the test organism. It is an ideal that is sometimes difficult to attain.

For assays of g.p.s.'s, in contrast to the assay media for antibiotics, etc., a synthetic medium must be devised so as to ensure compliance with requirement (3). This medium may include, for example, buffers, vitamin-free casein, glucose plus traces of amino acids, and vitamins other than the g.p.s.'s to be estimated.

A typical plate assay method is that for cyanocobalamin using *Escherichia coli*. This assay is used to illustrate a large plate quasi-Latin square design procedure in Example 6.

Tube assays for g.p.s.'s are the subject of Chapter 3.

A principle of both antibiotic and g.p.s. assays is described by Jerne and Wood (1949) as the "condition of similarity." That is to say, if the substance in the standard preparation that causes the characteristic response in the test subject is described as the *effective constituent*, then the response to the "unknown" test preparation must also be due only to the same effective constituent and be unmodified by other substances. In other words, the less potent of the two preparations (standard and test) that are being compared behaves as though it were a dilution of the other in an inert diluent.

It follows that when this principle is observed a change in experimental conditions, test organism, or response measured will not influence the true potency ratio between the two preparations. Any differences in estimated potency ratio would be attributable to experimental or random error only.

It also follows that in designing any assay procedure, it is necessary to take into account the possible influence of substances other than the effective constituent that may be present in either of the preparations. This is discussed further in Sections 1.5 and 1.6 and in Chapters 2–5, 8, and 9.

When the condition of similarity is truly applicable then the choice of test organism is dependent only on practical convenience, e.g., adequate sensitivity, sharpness of zone boundaries in plate assays, and slope of the response line. In practice, while very often the standard reference preparation may approach the ideal of being a dilution of the effective constituent in an inert diluent, the same may not be true of the test preparation. The latter may contain other active substances, either naturally occurring or as admixtures in pharmaceutical formulations. In such cases, the use of a test organism that is insensitive to the additional active constituent is necessary. Some examples are given in Chapter 5.

The practical importance of using the correct culture is clear.

1.3 Mechanization and Automation

Over a period of many years means have been sought to improve reliability and increase output of assay results. Mechanical aids have been developed for certain operations of manual assays and in more recent years partially and fully automated methods have been developed.

Mechanical aids include automatic diluters and media dispensers for liquid broth and molten agar. Coffey and Kuzel (1966) devised a machine for