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Analytical Survey

Replacement of microbiological assay by high-performance liquid chromatographic assay for antibiotics

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Abstract: The current position with respect to the expression of biological potency and chemical purity of antibiotics is reviewed. In particular, attention is drawn to the policies adopted in the USA which have been a source of confusion regarding the expression of biological potency. It is suggested that the opportunity provided by the introduction of high-performance liquid chromatography for the assay of antibiotics should be used to seek adoption of a more consistent approach for expressing antibiotic purity.

Keywords: *Assay of antibiotics; biological potency; microbiological assay; high-performance liquid chromatography (HPLC).*

Introduction

Recently a number of HPLC analyses have been proposed for the examination and assay of antibiotics; the antibiotics involved usually have a microbiological assay in their pharmacopoeial specification. In order to appreciate the difficulties which may arise through the replacement of microbiological assay by HPLC assay the concept of biological potency will be reiterated and the different forms of expressing potency (biological activity) explained. The aim of this article is to discuss the introduction of HPLC for antibiotics previously assayed microbiologically.

Microbiological assays have been used to measure the potency of those antibiotics which, because of their complex composition cannot be measured adequately by chemical or physical means. Often the structure of an antibiotic was not fully known so no realistic choice of a chemico–physical method could be made. Miles [1] in considering the concept of biological potency as applied to closely related antibiotics expanded upon the three essential requirements, originally proposed by Jerne and Wood [2] that must be satisfied for a valid biological assay. The first is that differences between responses in the several dose groups of an assay are wholly caused by differences in dosage and by random sampling. The second is that the response must be a determinable function of the

dose. Thirdly, the response of standard and test materials must be due to a single active principle. If more than one active principle is present, the proportions in the test and standard must be the same. This last requirement applies also to materials exerting an antagonistic or potentiating effect or affecting the stability of the active principle. When a biological assay is constructed with three dose levels each of standard and test and with a sufficient number of test organisms or measured responses in the several dose groups, its validity can be checked statistically and the potency ratio is obtained from the distance between the two dose–response lines. If the requirements are met, valid bioassays can be obtained and the potency, expressed in international units per unit of weight or volume, will be a measure of the content of the biologically active principle as well as of the biological activity.

In an excellent review on biological standardization Lightbown [3] emphasized that in practice the standard and test substances are rarely homogeneous. This was illustrated by reference to the difficulty of obtaining a valid microbiological assay of neomycin complex. The implication of this should always be remembered especially when biological and chemico–physical assays are being compared. As microbiological assays become reproducible and precise the material will be so pure that analytical control can be provided by chemical and physical methods. The use of HPLC for the analysis of antibiotics has allowed the separation of their constituent components thus providing the means for a specific assay. At present there are two distinct HPLC applications for antibiotics, quantitative assay and qualitative analysis.

Quantitative assays

There are now a number of HPLC assays in official use for antibiotics; in the British Pharmacopoeia [4] (Oxytetracycline Calcium) and in the United States Pharmacopeia [5] (Dactinomycin, Daunorubicin, Doxorubicin, Meclocycline Sulfosalicylate, Minocycline and Plicamycin). Proposals for more HPLC assays are being considered (Clindamycin [6], Doxycycline [7], Rifampin [8] and Tetracycline [9]) and examined (erythromycin). In these the assay result is expressed relative to a reference standard of defined purity or potency. The WHO Expert Committee on Biological Standardization [10] at its third session laid down the circumstances in which it considered it was valid to use three methods of designation of the potency of biological substances, i.e. units, gram-equivalents and grams. These circumstances were:

> "(1) Whether or not the active principles in biological substances have been characterized by chemical and physical means, the potency of preparations of such substances should be expressed in units whenever the active principle in both the standard preparation and preparations to be assayed may be heterogeneous;
>
> (2) The expression of potency in gram-equivalents is valid, although not always desirable, when the active principle in the standard preparation is known to be homogeneous and free from inert material, and the active principles in preparations to be assayed may be heterogeneous;
>
> (3) Designation of potency in grams is justified only when the active principle in the standard preparation is known to be homogeneous and free from inert material and when the active principle in the preparations to be assayed is known to be homogeneous. In such circumstances however, biological assay will be necessary only when expense prohibits the routine characterization of preparations by physical and chemical means."

The potency of a sample may be expressed in terms of a sample reference preparation as microgram-equivalents per mg indicating that 1 mg of the sample contains the activity of x μg of the standard preparation. This notation has meaning; if the unit of activity is defined as the activity of 1 μg of the reference preparation, then 1 unit is equivalent to 1 microgram-equivalent. Both are measures of activity. However, in the USA the

microgram notation has been based on a different concept; antibiotic potency is expressed not in microgram-equivalents but in micrograms per milligram. The potency is presented as a measure of weight content, not activity, and the weight measure is of a theoretical substance, not of a standard preparation. The difficulties of assessing the purity of the standard in absolute terms are considered relatively unimportant in the USA. If it becomes obvious that a mistake has been made, as when samples are found on assay to have a higher than theoretical content, then the potency of the master standard is adjusted in the light of the new knowledge, e.g. phenoxymethylpenicillin [11]. In several instances, as a result of the development of manufacturing and purification procedures preparations became available that contained more than 1000 micrograms of activity per mg, e.g. candicidin and vancomycin. It was then understood that such preparations had an activity equivalent to a given number of μg of the original reference standard. In most instances however, the microgram of activity is probably equivalent numerically to the μg (weight) of the pure substance. The main reason put forward in favour of microgram notation for expressing the potency of antibiotics is that the physician is used to prescribing on a weight basis and is confused by units. The procedure used in the British Pharmacopoeia in certain instances however, allows dosage in units of weight, so accommodating the physician, but exercises control of quality in terms of units of activity. The minimum permitted potency is expressed in units, e.g. 950 units per mg for oxytetracycline dihydrate. The dosage is expressed in weight of BP material. This method may be used when the antibiotic is homogeneous and when the minimum permitted potency represents a high degree of purity. The development of specific HPLC assays for many antibiotics means that it is now possible to define quality in terms of weight of the molecular entity.

During this transition phase different options have been chosen to calculate and express the result of an antibiotic HPLC assay. For Oxytetracycline Calcium BP the content is calculated as $C_{22}H_{24}N_2O_9$ from the declared content of $C_{22}H_{24}N_2O_9$ in Oxytetracycline Hydrochloride BPCRS. In those HPLC assays which are now official in the United States Pharmacopeia (Dactinomycin, Daunorubicin, Doxorubicin, Meclocycline Sulfosalicylate, Minocycline and Plicamycin) and those under consideration (Doxycycline and Clindamycin), the directions for preparing the Standard preparation imply, or explicitly state, that the μg per mg value on the label should be used in designating the concentration of μg in each ml of Standard preparation. Rifampin is an exception being defined on a weight basis.

Thus, the instructions for the assay of Doxycycline Hyclate USP state:

> "Calculate the potency, in μg of doxycycline ($C_{22}H_{24}N_2O_8$) per mg, of Doxycycline Hyclate taken by the formula:
>
> $$100\ (C/W)(ru/rs),$$
>
> in which *C is the concentration in μg per ml of doxycycline ($C_{22}H_{24}N_2O_8$) in the Standard preparation, W* is the quantity in mg of Doxycycline Hyclate taken to prepare the Assay preparation, and *ru* and *rs* are the peak responses obtained from the Assay preparation and the Standard preparation, respectively."

Solutions having a known concentration of about 1000 μg of doxycycline ($C_{22}H_{24}N_2O_8$) per ml are prepared. Thus, if the solution contains 1.200 mg per ml of a reference standard labelled as containing 865 μg per mg, its known concentration is 1038 μg per ml. In the above example the use of the term of potency, i.e. biological activity when the content of a chemical entity is intended, leads to confusion. In the USA this

may be dictated by the need to satisfy the legal requirements for potency, i.e. biological potency, even when a chemical assay is used. Instead a fresh approach should be adopted and the specification changed to a statement of minimum purity, i.e. content of a chemically defined substance, like any other chemically assayed material. Also in the above example the United States Pharmacopeia reinforces the idea that the potency of the reference standard defined in microgram-equivalents activity units is identical to its chemical content for the purpose of the HPLC assay. The tetracyclines present a special complication because no uniform policy was adopted in selecting the content of the base or salt to express the potency of the standard preparations. In some instances the potency of the standard is expressed in terms of theoretical base content, e.g. doxycycline, methacycline, minocycline, oxytetracycline and rolitetracycline. For others it is expressed in terms of a particular salt, e.g. chlortetracycline hydrochloride and tetracycline hydrochloride. Thus, the potency for theoretically pure tetracycline base is 1082 $\mu g\ mg^{-1}$. In the proposed HPLC procedure for Tetracycline this has led to the introduction of the factor P, defined as the potency in $\mu g\ mg^{-1}$ of the USP Tetracycline Hydrochloride RS.

The HPLC assay of plicamycin differs from the other assay procedures as the content of plicamycin is determined by reference to a calibration graph obtained using three different concentrations of the reference substance. Once an assay method has been validated, compliance with a monograph covers a narrow range of content relative to the concentration of the reference preparation so that any deviation from linearity should be unimportant within this range. The other HPLC antibiotic assays described are based on a direct comparison of equivalent test and reference preparations.

When an antibiotic containing several closely related biologically active components is assayed by HPLC, attempts may be made to convert the results into bio-equivalent potencies. In order to compare the bio-equivalent potencies of samples of erythromycin calculated from HPLC data with results of microbiological assay, allowance has been made for the antibacterial activity of erythromycins B and C [12] based upon their activity against *Staph aureus* ATCC 9144 relative to the main component erythromycin A [13]. Whilst such a relationship may hold in one laboratory it may not be reproducible in another or hold with another test organism as has been shown by Kibwage and coworkers [14]. Bio-equivalence is important for the interpretation of comparative results of a chemico–physical method and a biological method but it is irrelevant when determining the quality of an antibiotic. Ideally a good correlation should be demonstrated between the results of an HPLC assay and a microbiological assay, though for multicomponent antibiotics the correlation is usually poorer. This is often due to the different antimicrobial activities of the constituents of the antibiotic and therefore bio-equivalence factors are introduced to improve the correlation between the chemico–physical method and the biological method. Once an HPLC method of assay has been satisfactorily established there seems little advantage in expressing the result in terms of bio-equivalent potency, especially as biological potency of an antibiotic is relatively non-specific in the sense that only the inhibition of growth is being measured. In the case of erythromycin most samples contain 80% or more of erythromycin A which could readily be quantitated by HPLC and if required the proportion of erythromycins B and C could be determined. Antibiotic complexes which contain several major components and additional minor components, the proportions of which can vary with the source of manufacture, pose a more difficult problem for their quantitative assay and at present only the relative proportions are determined.

Qualitative analysis

HPLC is used to define the proportional composition of several antibiotic complexes. The analysis of Gentamicin Sulphate BP [15] is based on the pre-column derivatization technique of Freeman and co-workers [16]. The peak areas due to gentamicins C1, C1a, C2 and C2a are expressed as a percentage of the sum of the areas due to these four components, because the area responses per unit weight of the four are not identical [17]. The BP method does not indicate the actual composition of the gentamicin sample. In an attempt to compensate for this in changing from a paper chromatographic method with microbiological evaluation to HPLC the USP proposed [18] to change the limits of gentamicin C1a to 10–35% from 15–40% and the sum of gentamicins C2 + C2a to 25–55% from 20–50%, while the limit for gentamicin C1 would remain unchanged. The USP monograph for Bleomycin [19] specifies a range of proportional content for each of the two major components, bleomycins A2 and B2, with a minimum value for the sum of their contents; also there is a maximum for the proportion of bleomycin B4. Total peak area normalization is used to determine the relative amounts of the bleomycins specified in the sample being examined. The analysis of Tylosin BP [20] is based on the work of Fish and Carr [21]. The content of tylosin A and the sum of the content of tylosins A, B, C and D are determined by total peak area normalization of all peaks seen in the chromatograph. This is possible because the specific absorbances of the tylosin factors are virtually identical at the detection wavelength used. Peak normalization is used as reference substances for all the components are not available.

In the above examples it should be noted that the microbiological assay is currently retained to assess the overall potency of the antibiotics, since the HPLC is used only to determine the proportion of the components and not the actual amounts. Provided the specific absorbance of each component or derivatized component is identical it would be valid to use a single component as the reference substance, e.g. tylosin A. However, if the specific absorbances differ then the reference substance has to be either an artificial mixture of the individual components or a natural mixture of known composition and purity. In practice an artificial mixture may be difficult to obtain in sufficient quantity to serve as a reference substance. Once the relative composition of these antibiotic complexes has been defined it should be possible to agree to sum the individual components in order to define a quantitative minimum value of the total antibiotic content relative to the reference substance. This should be based on the quality of material in use rather than attempting to equate the HPLC assay with the existing microbiological assay. Now that the technical difficulty of separating the individual components by HPLC has been overcome the technique should be fully exploited to replace current microbiological assays.

Conclusions

HPLC has made an important contribution to the analysis and assay of antibiotics. The improvement in manufacturing technology has resulted in the production of highly purified antibiotics and the improved chemico–physical control, including chromatographic purity, of such materials has led to the deletion of the original safety test (test for abnormal toxicity) [22]. Unlike the responses in a microbiological assay those in an HPLC assay are considered not to be subject to random error, so confirmation of the validity of each estimate is thought unnecessary. The reproducibility of an HPLC system may be determined from the coefficient of variation of the peak responses of six replicate

injections: a value of 1.5% or less is acceptable to the USP. With modern injectors this figure can be very much reduced. For comparison the coefficient of variation of the zone of inhibition of growth of six replicate doses varied from 1.15 to 3.16% depending upon zone size (unpublished results). A more relevant comparison is the reproducibility of the assay method determined from the coefficient of variation of a number of replicate assays. For an HPLC method, six assays of a representative sample with a coefficient of variation of 2% or less are acceptable by the USP. In a recent collaborative microbiological assay of the International Standard for Kanamycin, coefficients of variation between 0.81 and 3.16% were obtained for six independent assays in different laboratories [23]. An independent assay was defined as including one separate ampoule of standard and test preparation. A properly designed microbiological assay should contain sufficient information to provide from its own internal evidence an estimate of potency with associated limits and an evaluation of the validity of the assay. Most microbiological assays have fiducial limits of ±3% or better when performed by competent laboratories. The advantages of the HPLC antibiotic assays over the microbiological are specificity, speed and precision though this last item is rather taken for granted. Nevertheless, HPLC is now widely accepted for the assay of antibiotics. In the UK, provision is made to allow a manufacturer to use a chemical or physical assay for an antibiotic instead of a microbiological assay [24] even when the latter is specified in a pharmacopoeial monograph. To prevent confusion which may arise through the introduction of HPLC assays for antibiotics it is suggested that the words reference substance and content be used for HPLC assays whilst standard and potency be confined to the microbiological assay.

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