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A comparative study of the microbiological assays currently available for nystatin raw material

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The classical agar diffusion and turbidimetric methods of assay for nystatin are compared with the more recently documented assays for this antibiotic which depend upon physicochemical measurement of the response of micro-organisms. Liquid nitrogen stored inocula were used throughout. It is concluded that the newer methods of assay are as reproducible and reliable as the agar diffusion and turbidimetric methods and that they are generally more sensitive. The choice between the assay methods compared can thus be based on speed, cost and sample through-put.

Polyene antibiotics are usually assayed microbiologically because of the poor correlation between uv spectrophotometric assays and biological activity (Trakhtenberg et al 1960; Gerke & Madigan 1961; Horvath & Koczka 1964; Elkouly et al 1973; Hamilton-Miller 1973a,b; Thomas 1976). In recent years attention has been paid to improving microbiological assays for these antibiotics. The application of cryobiology to the agar diffusion assay for nystatin gives zones of inhibition reproducible to within 1% (Cosgrove et al 1979). Inocula have been stored in liquid nitrogen for periods in excess of 5 years (Beezer et al 1976). Assays based on the loss of cytoplasmic constituents from susceptible cells as a consequence of nystatin action have also been reported. Such assay methods include the measurement of potassium ion loss (Ndzinge et al 1977), rubidium ion loss (Cosgrove 1978) and loss of ninhydrin-positive amine products (Smithler et al 1971; Evans & Bodnar 1973). A method, using flow microcalorimetry, that depends on changes in the heat output of yeast cells subjected to polyenes, has been reported (Beezer et al 1977a, b).

However, there are no reports that any of these physicochemical methods have been adopted routinely. This may reflect the reluctance of official organisations to move from the well-established classical methods of antibiotic assay, and also the general lack of comparative data and of standardization of the newer methods. A feature in the standardization of these methods may be the long-term storage of comparatively large amounts of uniform yeast cell inoculum in liquid nitrogen. The procedure of Beezer et al (1976) has been adopted for both the Rb^+ efflux assay (Cosgrove 1978) and the microcalorimetric assay (Beezer et al 1977a, b), and is largely responsible for the improvements to the agar diffusion assay (Cosgrove et al 1979).

The application of this type of uniform inoculum to the turbidimetric assay and the amino acid efflux assay for nystatin is feasible and enables a realistic comparison of all the nystatin bioassay methods. We have examined and compared the agar diffusion assay, the turbidimetric assay, the amino-acid efflux assay and the Rb^+ efflux assay. The latter is almost identical with the K^+ efflux assay (Ndzinge et al 1977) but is preferred because K^+, being an ion used routinely, could produce high background measurements and highten the chance of accidental contamination. The K^+ efflux assay (Ndzinge et al 1977) was compared with agar diffusion to assess the potency of three raw materials. In two cases the comparative results were favourable, but in the third the K^+ efflux method produced a result nearly 25% higher which could reflect a high K^+ ion content of the nystatin or contamination of the assay system. The report (Cosgrove 1978) on the Rb^+ efflux assay compared 10 raw material potencies with the diffusion method and all results derived by the two methods agreed to within $\pm 5\%$.

Assays based on efflux of Rb^+ and amino-acids, and on diffusion and turbidimetry were compared by the assay of four nystatin raw materials on two separate days to produce 8 results for each sample. The microcalorimetric method of assay is not included in the comparison because of the low sample through-put capacity and the relatively high cost of current equipment. However, figures for sensitivity,

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speed and reproducibility reported by Beezer et al (1977a, b) are included in the results section.

MATERIALS AND METHODS

Nystatin raw materials

Samples of four random batches manufactured by E. R. Squibb & Sons Ltd., were assayed in the method comparison. An internal Squibb reference standard (NY3) was used throughout.

Assay inocula

(i) *Saccharomyces cerevisiae* SC 1600 (Squibb Culture Collection) was used for the agar diffusion assay, the amino-acid efflux assay and the Rb^+ efflux assay. For the first two of these methods it was prepared as described by Beezer et al (1976) and for the Rb^+ method as described by Cosgrove (1978), in all instances storage was in liquid nitrogen.

(ii) *Candida tropicalis* ATCC 13803, used for the turbidimetric method, was stored under liquid nitrogen according to Beezer et al (1976).

Assay methodology

(i) Agar diffusion—as described by Cosgrove et al (1979). (ii) Rb^+ efflux—as described by Cosgrove (1978). (iii) Turbidimetric—essentially as described by Platt et al (1972) but with the use of liquid nitrogen-stored inoculum in place of daily prepared inoculum. (iv) Amino-acid efflux—modified from the methods of Smithler et al (1971) and Evans & Bodnar (1973) and carried out as follows: Nystatin standard material was dissolved in dimethylformamide (DMF) and diluted in 0·2M tris buffer ($NH_2.C(CH_2OH)_3$, adjusted to pH 7·5 with HCl) to 100, 75, 50, 25 and 12·5 units ml^{-1} (all solutions were compensated to contain 10% DMF). Samples of unknowns were similarly prepared at estimated values of approximately 75 and 25 units ml^{-1}.

An ampoule recovered from liquid nitrogen storage was thawed (3 min in 40°C water bath) and the contents subsequently diluted with 200 ml sterile saline. By means of a Fisons diluter (LFA/20 M.S.E. Crawley, Sussex) 0·1 ml of each standard and each sample solution was diluted with 4·9 ml of inoculum in polystyrene test tubes (Sterilin '144'). Each solution was assayed in triplicate and cell blanks and reagent blanks included with each assay. The loaded tubes were immediately placed in a shaking bath at 37°C for exactly 30 min. The tubes were then centrifuged at 3000 rev min^{-1} (M.S.E. Model GF6) for 5 min and the supernatants removed to clean containers. 2·5 ml of ninhydrin reagent (2 g ninhydrin and 300 mg hydrindantin dissolved in 75 ml methylcellosolve and diluted to 100 ml with 0·1M citrate buffer pH 5·0) was added to 3 ml of each supernatant, mixed and immediately heated in a water bath at 95°C for 8 min. After cooling, 5 ml distilled water was added to each solution and the colour intensity measured at 570 nm, against a reagent blank. A graph of log nystatin concentration against response was plotted for the standards and from this the potency of samples was determined.

RESULTS AND DISCUSSION

Table 1 summarizes the results of the raw material assays for each of the methods examined and Table 2 compares the characteristics of each of the assay methods (including the microcalorimetric method).

Table 1. Results of raw material assays used in the comparison of bioassay methodology for nystatin. The confidence interval ($P = 0·05$) is shown for each result.

Nystatin			
Batch 1	Batch 2	Batch 3	Batch 4
Agar diffusion			
4174±3·5%	5420±2·9%	4936±3·7%	5210±3·1%
Turbidimetric			
4352±5·5%	5270±4·8%	4810±6·1%	5365±4·2%
Amino acid efflux			
4348±5·0%	5254±4·5%	4791±5·2%	5125±4·8%
Rb^+ efflux			
4280±4·2%	5264±3·0%	5010±3·1%	5250±3·3%

The mean 95% confidence limits for the four raw materials (expressed as a percentage) were taken as the reproducibility of each assay. The lowest level of antibiotic concentration used in the determination of the standard response was taken as the sensitivity of the assay. It is possible in most instances to detect lower levels of nystatin, but the methods would require modification from those reported as the standard response would deviate from linearity at lower levels.

In an effort to detect any bias in any of the methods, all the assay results for each sample were combined and this figure used as a mean potency against which the results from the individual assay methods were compared.

There is good correlation between the methods and none were found to have a statistically significant bias.

Sensitivity

All the methods examined have the same order of sensitivity apart from the agar diffusion assay which is approximately 20–80 times less sensitive than the other methods.

Table 2. Characteristics of each nystatin bioassay method.

Sensitivity μ ml⁻¹	Mean 95% conf. int.	% Mean potency* 1	2	3	4	Inc. time (h)
		Diffusion				
20	± 3·3%	97%	102%	101%	99%	16–18
		Turbidimetric				
0·6	± 5·2%	101%	99%	98%	102%	5·25
		Amino acid efflux				
0·25	± 4·9%	101%	99%	98%	98%	0·5
		Rb^+ efflux				
1·0	± 3·4%	100%	99%	103%	100%	0·5
		Calorimetric				
0·5	± 3·0%	—	—	—	—	1·0

* Each assay result from Table 1 is expressed as a % of the mean result for each raw material determined by combining all the results from each of the assay methods.

Reproducibility

The range between the most reproducible (microcalorimetric) and the least reproducible (amino acid efflux) method is 3·0 to 5·2% and, therefore, for most purposes is of little significance.

Speed

All the methods require similar preparation times before incubation and it is the time taken for the incubation which accounts for the time differences between the methods. The assays involving physicochemical techniques are far shorter (30 min incubation for efflux assays; 60 min incubation for calorimetry) than the classical methods of agar diffusion (16–18 h incubation) and turbidimetry (5¼ h incubation). The development of an ion-selective electrode for use in the Rb^+ efflux assay (Cosgrove & Beezer 1979) might shorten this method even further (10 min incubation).

Sample through-put and requirements

The only assay having an instrumental limit to the number of samples that can be run concurrently is the microcalorimetric method. The present equipment only allows one sample to be run per hour and this does not include standards. Restrictions on the other methods are basically operator capacity and incubation space.

The physicochemical methods of assay require only buffer components as reagents (in addition to inoculum and antibiotic solution) while the agar diffusion and turbidimetric assays require a growth medium (agar or broth). The agar diffusion assay in its simplest form requires only a ruler, but can be semi-automated by use of an automatic plate reading device linked to a computer (Platt et al 1972); the Rb^+ efflux method requires either an atomic absorption spectrophotometer or an ion-selective electrode; the amino-acid efflux and turbidimetric methods require a photometer and the calorimetric method a flow microcalorimeter.

CONCLUSIONS

The use of liquid nitrogen-stored inocula has brought the reproducubility of all the assay methods for nystatin to within approximately 2% of each other. The newer physicochemical methods are as reliable as the classical procedures. All the methods are equally sensitive, apart from the agar diffusion method, but even this assay has a sensitivity within that required for most routine assay purposes.

Thus, speed, cost and sample throughput are now the important factors in deciding the most suitable assay for a given set of laboratory conditions. We see no reason why cryogenically stored inocula should not be used in the development and/or comparison of physicochemical and classical methods of assay for other antibiotics.

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