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The application of cryobiology to the microbiological assay of nystatin

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Improvements in the reproducibility of nystatin agar diffusion assays have been achieved by the use of liquid nitrogen stored inocula and deep frozen standard stock solutions. The overall percentage variability of the assay has been reduced from over 5% with daily prepared standards and inocula to around 1% with a frozen inocula and to 0·6% with a combination of frozen inocula and standards. The implications of these improvements in the standardization of nystatin assays, and microbiological assays generally are discussed.

The variation in the results obtained from routine microbiological assay procedures among different laboratories using the same standardized supply of antibiotic is a cause for concern. The results for the international standard for nystatin highlight the variation and difficulties inherent in a daily preparation of both inoculum and standard (Lightbown et al 1963). Whilst Arret & Eckert (1968) have stated that the 95% confidence range of an average microbiological assay is ±10%, the British Pharmacopoeia (1973) requirement is ±5% and currently any result within this range is considered normal variation and any result beyond this range is considered significantly different. The main contributory causes of this variation in supposedly comparable assays are variations in the inoculum, in the method of preparation of standard solutions of the antibiotic and also in available media (for example, see Freeman et al 1977).

Improvements in reproducibility in microbiological assays have been claimed for the use of cryogenically (under liquid nitrogen) stored inocula. These include *Sarcina lutea* for antibiotic assays (Stapert et al 1964), *Saccharomyces carlsbergensis* for vitamin assay (Tsuji 1966 a,b), *Saccharomyces cerevisiae* for polyene antibiotic assays by microcalorimetry (Beezer et al 1977 a,b), and by cell component leakage (Cosgrove 1978), and *Streptococcus faecalis* for the assay of chlorhydroxyquinoline (Cosgrove 1977). Only for the determination of this drug and for the polyene antibiotic assays has sufficient analytical detail been given to illustrate the quantitative improvements in reproducibility achieved by the use of such inocula.

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The preparation of standard solutions of the test antibiotic, e.g. nystatin, presents difficulties in that the drug is poorly soluble in aqueous media (Thomas & Peters 1977) and is labile with respect to temperature, pH and light (Thomas 1976). Nystatin also possesses detergent-like properties in that it foams whilst dissolving in dimethylformamide (DMF), the solvent of choice for initial solubilization (Thomas & Peters 1977).

We now report the results of large plate (8 × 8) two level, cavity type diffusion assays for nystatin in which (a) inocula of *Saccharomyces cerevisiae* were freshly prepared and standard nystatin solutions were made up daily, (b) inocula of *Saccharomyces cerevisiae* were stored at liquid nitrogen temperatures with daily preparations of standard nystatin solutions, and (c) inocula, as in (b), with standard solutions of nystatin prepared in DMF and stored at a variety of temperatures for periods up to 12 months.

MATERIALS AND METHODS

Inoculum

Saccharomyces cerevisiae SC 1600 (Squibb Culture Collection) prepared: (a) on a daily basis by the method described in The British Pharmacopoeia (1973); and (b) as a cryogenically (liquid nitrogen) stored inoculum (as described by Beezer et al 1976) essentially as follows: early stationary phase cells grown at 37°C were harvested from 8 litres of growth medium (soya peptone 5·0, yeast extract 1·5, lab-lemco 1·5, sodium chloride 20·0, glucose 12·5 g, distilled water 500 ml, 0·2M citrate buffer, pH 4, 500 ml), washed three times in sterile physiological saline and finally suspended in 250 ml saline.

Ampoules (2 ml, polypropylene screw-in cap

ampoules, '506', Sterilin Ltd) were rapidly charged with 1·8 ml of yeast suspension, dispensed from a Zippette (Jencons Ltd). Capped ampoules were held onto a cooling bar by uniformly spaced terry clips (1·2 cm diam.) which gripped the caps of the ampoules. Liquid nitrogen (initially 10 litres) was contained in an insulated stainless steel tray and the loaded cooling bars supported above the liquid nitrogen so that the ampoules were exposed to the vapour but not immersed in or even touching the liquid. The ampoules were rapidly released from the terry clips and plunged into liquid nitrogen after the temperature of the yeast suspension had passed −70 °C. The ampoules were removed from the freezing tray using pre-cooled forceps and placed into labelled canisters of a liquid nitrogen cryostat (CPV 35, British Oxygen Co. Ltd).

Immediately before use, sufficient ampoules were thawed in a water bath at 40°C for 3 min. Each ampoule was resuspended in 100 ml sterile physiological saline and treated, without further precautions, as a normal assay inoculum.

Assay medium

This was as described in the British Pharmacopoeia (1973).

Standard nystatin solutions

A Squibb internal standard of nystatin designated NY3 with a potency of 5735 units mg^{-1} was used throughout. After drying at 40°C under vacuum for 2 h, an amount containing 400 000 units of activity was dissolved in 100 ml DMF. Samples were stored at room temperature (20°C), 4°C (refrigerator), −20°C (deep freeze) and −196°C (under liquid nitrogen).

Assay procedure

Duplicate standards were prepared as described and diluted to 80 and 20 units ml^{-1} in 10% phosphate buffer (pH 6). DMF was added to the lower dilution to equate the DMF concentration of the two solutions. Samples were prepared similarly. All solutions were protected from light.

Assays were on 30 cm square glass-bottomed plates. 250 ml amounts of molten assay agar, cooled to about 45°C, were inoculated with a pre-determined (test plates) level of inoculum and poured into the sterile assay plates. When hardened, the agar on each plate had 64 wells (8 mm diameter) punched in it and solutions of standard and samples (0·05 ml) were filled into the wells in a latin square design. Duplicate standards, at the concentrations described in Table 3, and two different samples of nystatin, prepared at concentrations approximating those of the standard solutions, were used on each plate. Each assay was performed on 8 plates (i.e. 64 wells total) on each of 8 successive days. Loaded plates were incubated at 37°C for 18 h at which time the zones of inhibition were measured and the conventional statistical method (Finney 1964) for parallel line assays used for the analysis of the experimental results; the diameter of the inhibition zones being analysed in relation to the logarithm of the dose.

RESULTS

A cryogenic inoculum for the assay of nystatin has been used by the authors for a period in excess of 18 months. As shown in Table 1, the viability of the inoculum has remained constant throughout this period and the resultant zones of inhibition have been relatively constant in diameter.

Table 1. Viability and assay response of a liquid nitrogen stored inoculum of *Saccharomyces cerevisiae* in a two level agar diffusion assay for nystatin over an 18 month period using a freshly prepared nystatin standard.

Length of inoculum storage (months)	Viability %	Average inhibition zone size (mm)	
		80 u nyst.ml^{-1}	20 u nyst. ml^{-1}
Initial*	91	18·8	14·3
1	88	18·8	14·2
2	90	18·5	13·8
3	92	18·6	14·2
6	91	18·6	14·1
9	90	18·6	14·0
12	90	18·7	14·2
18	91	18·5	14·1

* i.e. immediate recovery after freezing.

The results of the storage of nystatin standard solutions at a concentration of 4000 units ml^{-1} in DMF at various temperatures are shown in Table 2. It can be seen that the standard is stable at −196° and −20°C for at least 12 months. At higher temperatures there is a significant loss of potency with time. The overall percentage of variation of zone size is approximately 5% with a freshly prepard standard and inoculum (Group A—Table 3), around 1% with a liquid nitrogen stored inoculum (Group B—Table 3), and <0·7% with a cryogenically stored standard and inoculum (Group C—Table 3). The mean diameters of the inhibition zones observed with cryogenically stored inocula are

Table 2. Stability of standard nystatin solutions (4000 u ml^{-1}) in DMF at various temperatures. Results expressed as % freshly prepared standard. Liquid nitrogen stored inoculum used throughout.

	RT (20°C)	4°C	−20°C	−196°C
1 Week	99	99·5	101·5	100
1 Month	94	100	100	100
3 Months	<90	96·5	99	99
6 Months	—	<90	99	99
9 Months	—	—	101	100
12 Months	—	—	99	98

significantly smaller than those observed with freshly prepared inocula.

Table 4 shows data selected at random for 30 batches of nystatin raw material that have been routinely assayed. The introduction of the liquid nitrogen stored inocula reduced the mean 95% confidence interval on 8 results from 5·65% to just over 2%.

DISCUSSION

Potency results for unknown samples are based upon interpolation of the results obtained in a log dose vs inhibition zone diameter plot (Kavanagh 1972). Reproducibility in potency assays will be a reflection in part, of the constancy of the slope of this line. The data (Group A—Table 3) illustrate that the zone diameters obtained with a freshly prepared standard and inoculum have a reproducibility which is low. The reproducibility of the slope of the log dose-response line derived from these data is poor. The results quoted (Groups B & C—Table 3) show that the use of a liquid nitrogen-stored inoculum substantially improves the replication of nystatin agar diffusion assays (the slope of the dose-response lines are much improved in reproducibility). Conditions have also been defined for the long-term storage of nystatin standard stock solutions and the combination of these with the frozen inoculum enables the prediction of inhibition zone sizes to within 1%. The significant improvement in reproducibility achieved by use of the combination of a stored standard and inoculum over the stored inoculum alone, may represent limiting values, since the error in the manual measuring of inhibition zones is probably now the largest factor in the confidence intervals quoted and only improvements in the methods of measurement will reduce this figure further.

Table 3. Average size of inhibition zones presented as a mean value for the high (80 u ml^{-1}) and low (20 u ml^{-1}) dose from eight individual assay plates on each of eight separate days (i.e. each figure represents 8 days testing with 64 zones per day). Each result is shown with its 95% confidence interval ($P = 0·05$).

Group	Standards	Inoculum	Average zone size (mm) 80 units ml^{-1}	20 units ml^{-1}
A	Fresh	Fresh	20·6 ± 1·05 (5·1%)	15·8 ± 0·79 (5·0%)
B	Fresh	Liq.N_2	18·7 ± 0·16 (0·86%)	14·2 ± 0·15 (1·06%)
C	−20°C	Liq.N_2	18·8 ± 0·12 (0·64%)	14·2 ± 0·08 (0·56%)

Table 4. Assay results, selected at random, (performed as described in the heading of Table 3) for batches of nystatin raw material assayed: (i) Group A—before the use of liquid nitrogen stored inoculum or deep-frozen standards; (ii) Group B—with liquid nitrogen stored inoculum and freshly prepared standards, and, (iii) Group C—with liquid nitrogen stored inoculum and deep-frozen standards. Each result is shown with its 95% confidence interval ($P = 0·05$) and is the mean of 8 individual results.

Group A	Group B	Group C
4186 ± 5·5%	5249 ± 2·0%	5058 ± 1·8%
5039 ± 4·6%	5160 ± 2·3%	5206 ± 1·6%
3714 ± 7·0%	5232 ± 3·2%	5275 ± 2·6%
5306 ± 3·6%	4985 ± 1·5%	5202 ± 2·9%
5256 ± 5·4%	4969 ± 1·9%	5035 ± 2·6%
5613 ± 5·2%	4954 ± 3·2%	5370 ± 2·8%
5705 ± 5·8%	5292 ± 2·3%	5619 ± 1·6%
5497 ± 6·9%	5552 ± 1·9%	5006 ± 2·6%
5594 ± 7·1%	4648 ± 2·1%	5290 ± 2·0%
6087 ± 5·4%	5213 ± 1·1%	5142 ± 1·8%

The daily reproducibility achieved with a cryogenically stored inoculum compared with a freshly prepared inoculum, is thought to be related to the method of inoculum preparation. The cells for the liquid nitrogen-stored inoculum are grown in broth culture and harvested at the early stationary phase; thus most will be of a similar age. In contrast the cells for the freshly prepared inoculum are grown on agar slopes and will consequently consist of a population of mixed ages and it is this factor which is thought to be responsible for the daily variation in Group A data (Table 3).

These improvements to the assay system raise the possibility of the establishment of new criteria for international standards in that it is now possible to maintain both standard and inoculum over extended periods of time. The existence of such standards would permit the calibration of new batches of nystatin and inocula to be used as secondary standards (cf. the variation in interlaboratory tests to establish the international standard for nystatin (Lightbown et al 1963)).

Published evidence (Hossack 1972) suggests that liquid nitrogen stored inocula are presently distri-

buted worldwide. It is however conceded that unless care were taken it would be possible to introduce bias into such assays based upon one stored standard solution.

Experience with this assay system (unpublished findings) has led to increased reliability, a reduced repeat rate, increased confidence between laboratories, a substantial saving of time, constant availability of inocula, and reduced need for assay supervision. The benefits of the use of cryogenically stored inocula have also been described (without experimental detail) by Hossack (1972).

The reproducibility accessible through the use of liquid nitrogen stored inocula in combination with standard stock solutions permits consideration of a reduction in the number of replicates required to give a defined overall reproducibility in the assay and also brings the assay to within the same levels of precision obtained using other methodological forms of assay, e.g. microcalorimetry (Beezer et al 1977 a,b), atomic absorption spectrophotometry (Cosgrove 1978).

In conclusion, we would advocate the examination of other antibiotics for storage as assay stock solutions, as their use, in combination with frozen assay inocula, produces a reproducibility of response hitherto not met in microbiological diffusion assays.

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