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The International Standard for Nystatin

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At the request of a WHO Expert Committee on Biological Standardization, a batch of nystatin, comparable in purity to material used therapeutically, was submitted to an international collaborative study in nine laboratories in six countries. In this study the material was assayed by the plate diffusion method (and in one laboratory also by a turbidimetric method) against the United States Pharmacopeia Reference Preparation of Nystatin. On the basis of the results obtained the material has been established as the International Standard for Nystatin, and the International Unit of Nystatin is defined as the activity in 0.000333 mg of the International Standard.

At its meeting in Geneva in September 1957, the WHO Expert Committee on Biological Standardization (1958) asked the Department of Biological Standards of the National Institute of Medical Research, London, to obtain an adequate quantity of nystatin and examine its suitability for use as an international reference preparation. A sample of the substance was obtained in 1957 and established in 1958 as the International Reference Preparation of Nystatin (WHO Expert Committee on Biological Standardization, 1959). At the meeting of the WHO Expert Committee on Biological Standardization held in 1960, it was reported that, as requested in 1959, the National Institute of Medical Research had arranged for an international collaborative study of the material; the Committee accordingly authorized the Institute "to establish the material as the international standard for nystatin and to define the international unit with the agreement of the participants in the collaborative assay" (WHO Expert Committee on Biological Standardization, 1961).

PROPOSED INTERNATIONAL STANDARD

The material, consisting of 400 g of a single batch of nystatin (lot No. 15287-040), was obtained through the generosity of E. R. Squibb & Sons, United States of America. The following data were supplied by the manufacturer:

Potency: 2855 units/mg after drying at 40°C for 2 hours *in vacuo*;
Moisture: 2.32%;
pH (3% suspension): 7.1;
Specific rotation at 25°C; + 7.74;
Residue on ignition: 7.24%.

The material was kept in its original sealed containers at —10°C until February 1959, when it was distributed into approximately 3500 ampoules, each containing approximately 75 mg. The ampoules were stored over P_2O_5 *in vacuo* at + 4°C for 13 days, the P_2O_5 being changed once. The ampoules were constricted, stored *in vacuo* over P_2O_5 for a further 18 days, filled with pure dry nitrogen and sealed. After being sealed the ampoules were tested for pinholes and leaks and were then stored at —10°C. A portion of the distributed material is being used as the United States Pharmacopeia (USP) Second Reference Preparation of Nystatin.

The moisture content of the material in the ampoules was measured by heating at 56°C at 0.05 mm Hg for 24 hours and was found to be 1.4% w/w. There was no significant difference between the moisture contents of 10 separate ampoules chosen at random.

When exposed to an atmosphere of 50% relative humidity, the material in the ampoule picked up approximately 1.0% moisture in 10 minutes and approximately 7.0% moisture in 5 hours.

THE USP REFERENCE PREPARATION OF NYSTATIN

Thirty vials, each containing 200 mg of the USP Reference Preparation of Nystatin, were made available and distributed for the collaborative assay by Dr Lloyd C. Miller, Director of Revision of the Pharmacopeia of the United States of America. This material was issued in the form in which it was dispensed by the USP, and collaborating laboratories

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were requested to follow the instructions given on the label. These directed that the material should be dried *in vacuo* at 40°C for 2 hours before using.

THE COLLABORATIVE ASSAY

The two preparations (three vials of the USP Reference Preparation and eight ampoules of the proposed International Standard) were sent to 9 laboratories in 6 different countries which agreed to take part in the collaborative assay. Throughout this report these laboratories are referred to by arbitrary numbers only; but a full list of names and addresses is given in the Annex.

In the memorandum which accompanied the preparations no particular assay method was specified, but participants were asked if possible to perform six separate assays. Each assay was to contain sufficient information to provide, from its own internal evidence, an estimate of potency and confidence limits to that estimate, and also evidence of linearity and parallelism of the log-dose/response lines.

Preliminary comparative assays of the proposed International Standard carried out at the National Institute for Medical Research had suggested that there was a between-container variation in one of the two preparations. Further assays carried out by two laboratories in the United States of America did not confirm this, but collaborating laboratories were asked to compare the two preparations in such a way that any inter-ampoule or inter-vial variation could be detected.

Two arrangements were suggested which would allow possible variation between containers to be evaluated:

	Assay	*USP Reference Preparation*	*Proposed International Standard*
Design I	1	Vial 1	Ampoule 1
	2	,, 1	,, 2
	3	Vial 2	Ampoule 3
	4	,, 2	,, 4
	5	Vial 3	Ampoule 5
	6	,, 3	,, 6
Design II	1	Vial 1	Ampoule 1
	2	,, 1	,, 2
	3	,, 2	,, 1
	4	,, 2	,, 3
	5	,, 3	,, 2
	6	,, 3	,, 3

It was indicated that Design I could be split over three days as indicated by the dotted lines, whereas Design II (which was the better design from a statistical point of view) had to be completed in one day.

RESULTS

The results of a total of 330 assays, comprising 282 assays by the plate diffusion method and 48 assays by the turbidimetric method, were received from the nine collaborating laboratories. The number of assays performed and the method used by each laboratory are shown in Table 1.

STATISTICAL METHODS OF ANALYSIS

The results of each experiment were analysed by the standard method for parallel-line assays, using analysis of variance appropriate to the design of each assay.

In most of the assays the diameter of the zone of inhibition of growth was used as the response metameter. In Laboratory 6, two dose levels were used for both the reference and the test preparation; as this laboratory considered the square of the diameter the most suitable response metameter, assays performed in this laboratory were analysed by taking the squared diameter as the metameter. For Laboratory 4, whose results were analysed at an early stage, the analysis made of the zone diameters revealed a considerable number of cases of curvature of the regression lines. A new analysis was carried out, using the squared diameter as the response metameter. This analysis reduced the number of cases of non-linear regression, but the estimated potency of the proposed International Standard material and its confidence limits remained practically unchanged. A closer examination of the analysis using zone diameters showed that significant non-parallelism or curvature of regression lines had been brought out mostly by the fact that in these assays the error variance was much smaller than in the other assays which showed no significant departures. For these reasons the data from all the other laboratories were analysed by using the zone diameter itself, although slight improvement might have been made in some cases if the squared zone diameter had been used as the response metameter. The potencies reported here for Laboratory 4 are those calculated on the basis of the squared zone diameters. Assays showing statistical invalidities were not automatically excluded from the final evaluation.

TABLE 1
NUMBER AND TYPE OF ASSAYS PERFORMED IN DIFFERENT LABORATORIES

Laboratory No.	Type of assay	Design of assay	Test organism	Number of assays performed	Number of assays used for statistical evaluation
1	Diffusion	3 + 3	*Saccharomyces cerevisiae* (A. Coll. 9763)	11	11
2	"	3 + 3	*Saccharomyces cerevisiae* (A.C.T.C. 97632)	18	18
3	"	3 + 3	*Saccharomyces cerevisiae* (Strain not specified)	48	48
	Turbidimetric		*Saccharomyces cerevisiae* (Strain not specified)	48	48
4	Diffusion	3 + 3	*Candida albicans* (Laboratory strain)	18	18
5	"	3 + 2	*Saccharomyces cerevisiae* (Squibb No. 1600)	67	67
6	"	2 + 2	*Saccharomyces cerevisiae* (Squibb No. 1600)	48	47
7	"	3 + 3	*Saccharomyces cerevisiae* (Squibb No. 1600)	30	30
8	"	2 + 2	*Candida albicans* (Strain not specified)	36	16
9	"	3 + 3	*Saccharomyces cerevisiae* (M 53)	6	6
			Total	330	309

The results of 21 assays were not included in the final evaluation of the potency of the proposed International Standard material. Eighteen of these were assays performed in Laboratory 8 by using the USP Reference Preparation without drying. As no reliable estimate of the moisture content of USP vials was obtained by this laboratory, an attempt was made to adjust the results on the basis of the data available from Laboratory 6. However, the adjusted potency estimates turned out significantly different from the potency values obtained in the later assays done in Laboratory 8 by using the USP Reference Preparation after drying. The other three assays discarded from the final analysis include one assay in which too many observations were missing (Laboratory 6), and two assays which produced potency estimates far different from those obtained in the other assays performed in the same laboratory (Laboratory 8) and in which there was highly significant departure from parallelism of the dose-response lines.

When all the individual assays were analysed and the potency estimates and their weights computed, the homogeneity test (Humphrey, Mussett & Perry, 1953) by means of χ^2 was applied to study whether the potency estimates obtained in the same laboratory were homogeneous, i.e., whether the differences among the estimates were of the same order as expected from the internal evidence of individual assays. The potency estimates obtained within all laboratories, except Laboratory 9, were very heterogeneous. There was no evidence of heterogeneity in the results from Laboratory 9 and hence a combined estimate of potency was obtained in this case by computing the weighted geometric mean according to the usual procedure (Humphrey, Mussett & Perry, 1953). For the other laboratories this method could not be used because of the heterogeneity. In each of these instances, therefore, the geometric average potency was calculated from the results of the individual assays performed in each laboratory. The within-laboratory variance was computed for the average potency on the basis of the spread of the frequency distribution of potency values obtained in each laboratory. The results are shown in Table 2, columns 3, 4 and 5.

The combined potency estimate obtained for each laboratory varied considerably from one laboratory to another ($\chi^2 = 186.2$; $P<0.001$). The between-laboratory variance was therefore computed and the weight of each combined potency estimate was determined (Bliss, 1952) by taking the reciprocal of the total variance, i.e., including both within- and between-laboratory variances, as shown in Table 2, columns 6 and 7. One combined estimate of potency for the entire results was determined by computing the weighted average logarithmic potency using the weight values shown in Table 2, column 7. A value

TABLE 2

SUMMARY RESULTS OF DIFFUSION AND TURBIDIMETRIC ASSAYS OF PROPOSED INTERNATIONAL STANDARD FOR NYSTATIN

Laboratory No. (1)	Number of assays (2)	Geometric average potency (units/mg) (3)	Average log potency [a] (M) (4)	Within-lab. variance (S_M^2) (5)	Total variance ($S_M^2 + S_m^2$) (6)	Weight (7)	Factors studied in analysis of variance of potencies obtained within laboratories [b] (8)
1	11	2 719	3.43440	0.000 143 96	0.000 789 54	1 267	Date,* vial, cloud cover, time difference between preparation of proposed IS and USP solutions *
2	18	2 920	3.46536	0.000 004 05	0.000 649 63	1 539	Weighing, vial, ampoule
3a [c]	48	3 202	3.50543	0.000 022 93	0.000 668 51	1 496	Date, operator*, vial *, ampoule
3b [c]	48	3 290	3.51723	0.000 038 16	0.000 683 74	1 463	Date,* vial/ampoule *
4	18	2 752	3.43968	0.000 030 21	0.000 675 79	1 480	Vial, ampoule
5	67	2 927	3.46647	0.000 020 74	0.000 666 32	1 501	Operator,* date/ampoule *
6	47	2 973	3.47318	0.000 039 40	0.000 684 98	1 460	Vial/ampoule *
7	30	3 015	3.47935	0.000 008 63	0.000 654 21	1 529	Date,* operator *
8	16	2 834	3.45244	0.000 017 64	0.000 663 22	1 508	Date,* vial/ampoule *
9	6	2 969	3.47267	0.000 013 82	0.000 659 40	1 517	(Results were homogeneous)

[a] The over-all average log potency for the nine laboratories obtained by weighting the values in column 4 by those in column 7 is 3.47110, corresponding to an over-all average geometric potency of 2959. The total weight for this value is 14 760.

[b] An asterisk indicates that the factor was found to be a source of statistically significant variation of the potency estimate ($P < 0.05$). " Vial/ampoule " or " date/ampoule " means the combination of two factors which could not be isolated.

[c] 3a relates to the diffusion assays and 3b to the turbidimetric assays performed in Laboratory 3.

of 2959 units/mg was obtained in this way with confidence limits ($P = 0.95$) 2851-3071 units/mg, the weight of this estimate being 14.8×10^3.

For comparison, the simple geometric average for the potencies of the 309 assays, from all laboratories but without weighting, was calculated and found to be 3015 units/mg, with confidence limits ($P = 0.95$) of 2983-3048 units/mg. The weight of this grand mean was 178×10^3.

The frequency distribution of the 309 potency values is shown in the figure opposite.

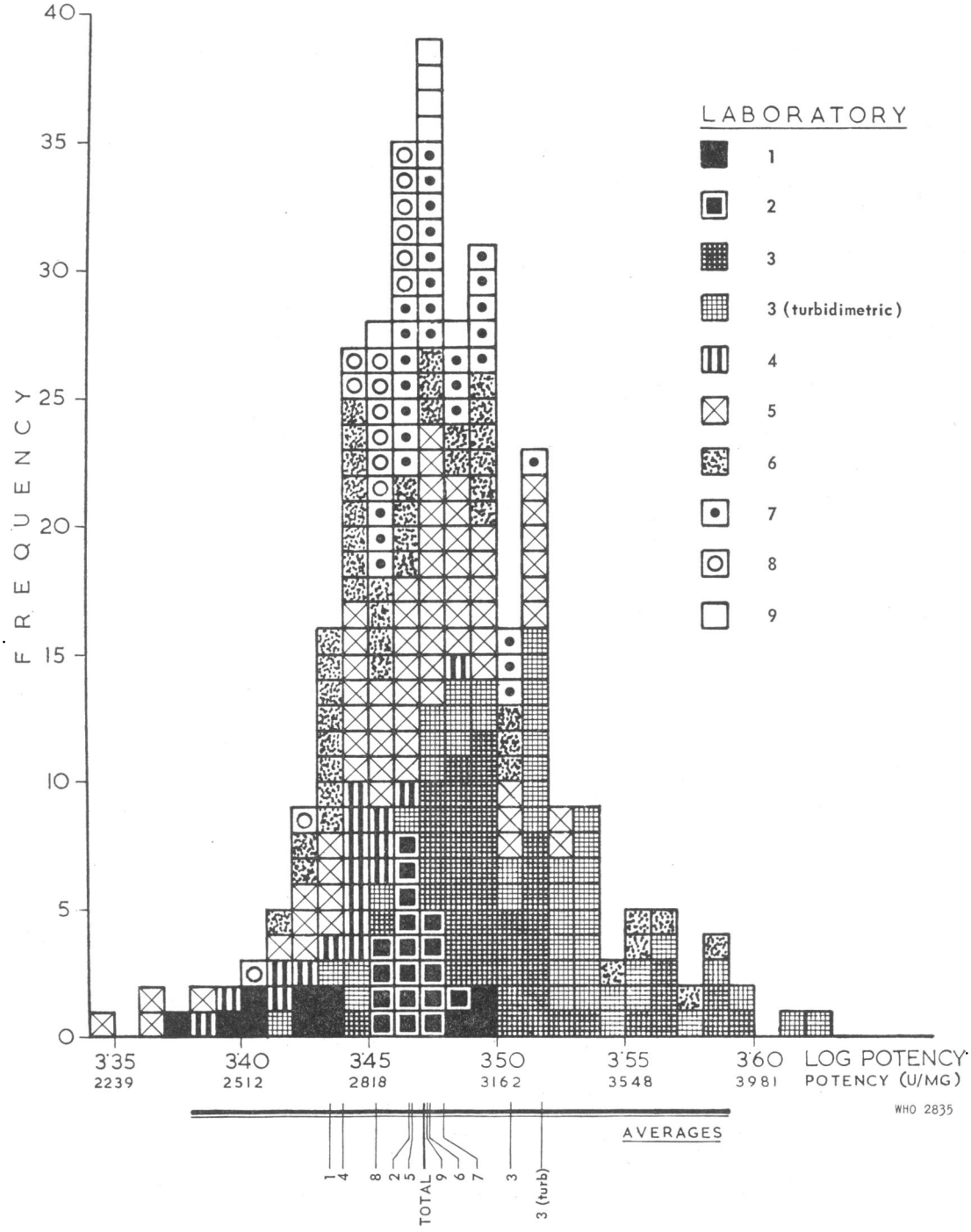
Where possible the sources of variation of individual potencies within each laboratory were studied by analysis of variance. The factors examined were: between days, operators, USP vials, proposed Standard ampoules, etc., as shown in Table 2, column 8. Those factors found to be significant sources of variation are marked there with an asterisk.

DISCUSSION

Seven laboratories used an assay design which enabled linearity of the dose-response lines to be assessed and with each of these laboratories a varying number of assays showed significant curvature, at the 0.95 level, the total number of such assays for these seven laboratories being 23 out of 198. Significant departure from parallelism of the dose-response lines was observed in a number of assays, in all except one laboratory (No. 2). Such assays are statistically invalid but are to be expected when impure materials are compared in a biological assay. They reflect a heterogeneity of the materials themselves and demonstrate the difficulties of standardizing the biological test system even within a single laboratory.

The two preparations which were compared in this study had approximately equal potency in units/mg, but both were of a low order of purity. Nystatin with a potency of 5000 units/mg has been described which would indicate that both the USP Reference Preparation and the proposed International Standard were not more than 50% pure. The difficulties which arise in the comparison of such preparations have been pointed out by Miles (1952) and these are illustrated in the results of previous collaborative assays of antibiotics and other biologically active materials (Lightbown, 1961). In the light of past experience the heterogeneity of potency estimates obtained by different laboratories and within laboratories in this study is not surprising. The inability to standardize conditions in biological assays in this instance is well illustrated by the heterogeneity

FREQUENCY DISTRIBUTION OF THE LOG POTENCIES OF THE PROPOSED INTERNATIONAL STANDARD FOR NYSTATIN OBTAINED IN DIFFERENT LABORATORIES



of potency estimates which was found within each laboratory except No. 7 and No. 9. In the latter case some statistically invalid assays were obtained, but were not submitted for the general analysis.

The analysis of variance of assays performed within each laboratory failed to indicate any general reason for their heterogeneity (see Table 2). The suspected variation between vials or between ampoules was not clearly confirmed, although vials were a major source of vial/ampoule variation in Laboratory 3, and either vials or ampoules contributed significantly to the heterogeneity in four other laboratories.

Preliminary experiments in Laboratory 6 showed that exposure of the prepared solutions to daylight during the course of the assay affected the results in a variable fashion. It was confirmed that solutions of both the USP Reference Preparation and the proposed International Standard were inactivated by exposure to light, but at different rates. The extent of the inactivation by daylight was affected by the amount of cloud cover; the wave-length of the inactivating light was not ascertained, but no protection was afforded by ordinary glass. When the assays were performed in a darkened room protected from daylight, the variation between assays was greatly reduced, but a significant departure from parallelism was obtained in nearly all assays.

Data were available regarding the cloud cover in Laboratory 1 at the time of the different assays, but this factor did not prove to be significant. However, the time difference between preparation of the solutions of the USP Reference Preparation and the proposed International Standard which varied from assay to assay was a significant source of variation. If the solutions are prepared under conditions where they may be inactivated by light, the order in which they are prepared would be expected to affect the potency obtained. Unfortunately, data were not available regarding the time sequence of the preparation of the solutions in the other laboratories.

The possibility that there may be a fundamental difference in slope between the two preparations was examined, as also was the possibility of a relationship between the slope of the regression line and the potency computed for each individual assay. The results of this study are shown in Table 3. The slope of regression lines tended to be steeper for the proposed Standard in Laboratories 3, 4 and 6 (assays on 7 July 1960), while it tended to be steeper for the USP Reference Preparation in Laboratories 2 and 6 (assays on 4 October 1960) and 7. However, the conditions of assay regarding exposure to daylight in each laboratory are not known, and it is not possible to draw any conclusions. For the whole study

TABLE 3
NUMBER OF ASSAYS AND AVERAGE LOG RELATIVE POTENCY OF PROPOSED INTERNATIONAL STANDARD, IN RELATION TO THE SLOPE OF DOSE-RESPONSE REGRESSION LINES

Laboratory No.	Steeper line for USP Reference Preparation		Same slope for USP Reference Preparation and proposed International Standard		Steeper line for proposed International Standard	
	Number of assays	Average log relative potency	Number of assays	Average log relative potency	Number of assays	Average log relative potency
1	5	−0.0336	—	—	6	−0.0369
2	10	0.0084	3	0.0128	5	0.0105
3	15	0.0348	—	—	33	0.0515
4	5	−0.0289	—	—	13	−0.0307
5	36	0.0142	—	—	31	0.0070
6 (7.7.60)	6	0.0324	—	—	18	0.0170
6 (4.10.60) [a]	22	−0.0161	—	—	1	−0.0251
7	18	0.0108	3	0.0201	9	0.0057
8	4	0.0063	2	−0.0052	10	−0.0050
9	3	0.0040	—	—	3	0.0013
Total	124	0.0323	8	0.0277	129	0.0039

[a] Assays performed in a darkened room.

the slope was as frequently steeper for the USP Reference Preparation as for the proposed International Standard.

It can be seen from the figure on page 91 that potencies from individual assays varied over a very wide range—namely, from 2191 to 4203 units/mg. As might be expected, laboratories which performed the greatest number of assays obtained the widest range in potency values. Considering all the assays together, the distribution of log potencies is fairly normal, although Laboratory 3, particularly with its turbidimetric assays, accounts for a large proportion of the high values. All laboratories used a strain of *Saccharomyces cerevisiae* as test organism except Laboratories 4 and 8, which used *Candida albicans*. All but one of the assays from these two laboratories gave potencies less than the over-all mean. The figure illustrates clearly the differences in potency which can be expected when different laboratories compare the same material against the proposed Standard. These differences may be large but they are practically unavoidable with materials of this degree of purity. Since the proposed International Standard will be used in the assay of material of a comparable purity, and since there is no reason to believe that any other similar sample would give more consistent results, the proposed Standard is established as the International Standard for Nystatin. With the agreement of the collaborating laboratories, the potency of the International Standard is defined as 3000 International Units (IU) per mg. As is customary with all international standards this is the potency of the material as it is in the ampoules without further drying.

CONCLUSION

In accordance with the request of the WHO Expert Committee on Biological Standardization (1961), the International Reference Preparation of Nystatin has been established as the International Standard for Nystatin on the basis of an international collaborative assay. The potency of the International Standard is defined as 3000 IU/mg. The International Unit of Nystatin is therefore defined as the activity in 0.000333 mg of the International Standard.

Annex

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RÉSUMÉ

En 1958, le Département des Etalons du National Institute for Medical Research, à Londres, a été autorisé par un Comité OMS d'experts de la Standardisation biologique, à établir un Etalon international de Nystatine. Un lot de 400 g de nystatine, obtenu en 1957, a été réparti en ampoules de 75 mg, complètement desséché, les ampoules remplies d'azote sec et scellées. Ce lot, destiné à servir de Préparation internationale de référence a été soumis à des essais biologiques — et comparé à la Préparation de référence de la pharmacopée des Etats-Unis — dans neuf laboratoires de six pays. Les résultats de 330 essais (soit 282 par la méthode des plaques et 48 par opacimétrie) ont été examinés et 21 d'entre eux éliminés.

Des écarts très importants ont été constatés entre les résultats individuels, allant du simple au double, ce qui n'a rien d'étonnant pour une substance dont la pureté n'est que de 50%. Cet état de choses ne pouvant être modifié actuellement, le lot proposé a été adopté comme Préparation internationale de référence de Nystatine. D'accord avec les laboratoires ayant pris part à l'essai comparatif, la Préparation internationale de référence de Nystatine a été définie comme contenant 3000 unités/mg, et l'Unité internationale de Nystatine comme correspondant à l'activité de 0,000333 mg de la Préparation internationale de référence.

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