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The second international standard for polymyxin B *

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Since supplies of the first International Standard for Polymyxin B were exhausted, it was replaced by a second international standard the potency of which was estimated from the results of a collaborative assay carried out by 5 laboratories in 4 countries. The wide variations in the results probably resulted from difficulties experienced in handling the first international standard. The potency finally agreed upon by the collaborating laboratories, on the basis of the overall mean values obtained after rejection of the most discrepant assays, was 8 403 IU/mg. That value was accepted by the WHO Expert Committee on Biological Standardization (1970), which consequently defined the International Unit of polymyxin B as the activity contained in 0.000119 mg of the second international standard.

In its twenty-first report, the Expert Committee on Biological Standardization (1969) noted that stocks of the first International Standard for Polymyxin B, established in 1955, were depleted; that material suitable for its replacement had been obtained by the National Institute for Medical Research, London; and that a collaborative assay was in progress. The properties of the material and the results of the collaborative assay are described below.

MATERIALS FOR THE STUDY

The proposed second international standard for polymyxin B

A sample of about 400 g of polymyxin B sulfate, Batch No. 33274, was generously made available by Burroughs Wellcome & Co., Dartford, Kent, England, through the good offices of Dr G. A. Stewart. The sample was received at the National Institute for Medical Research, in May 1967, in a single container, and was stored at −20°C in the dark, protected from moisture.

The following data were supplied by the manufacturer.

description: white, almost odourless powder
stability: very soluble in water, yielding a bright colourless solution
identity tests: complies with tests A, B, and C given under "Identification" in the British Pharmacopoeia, 1963 (page 629)
acidity: pH of 2% w/v solution=6.6
loss on drying at 60° *in vacuo*: 3.64%
sulfated ash: 0.05%
potency: estimated potency=7 743 IU/mg; limits of error 98.1–101.9% of estimated potency (P=0.95)
pyrogen test (USP): passed (20 000 IU per kg of body weight in rabbits; summed response for 3 rabbits=0.8°C; no individual response greater than 0.59°)
toxicity test (USP): passed (600 IU in 0.5 ml of 0.9% saline injected i.v. into each of 5 mice; no mice died)
toxicity test (BP): passed (600 IU in 0.2 ml of water injected i.v. into each of 5 mice; no mice died)
composition: hydrolysis and GLC of the resulting fatty acids revealed the following composition: polymyxin B_1 sulfate, 77.5%; polymyxin B_2 sulfate, 21.4%; unidentified product yielding an unknown fatty acid, 1.1%

Preliminary attempts to distribute the material into ampoules as a dry powder were unsatisfactory. After exhaustive drying, it was found impossible to seal the ampoules by fusion of the glass without the charring of particles of powder in the vicinity of the seal. The material was therefore dissolved in water, distributed into ampoules as a solution, and freeze-dried. The details of this procedure are as follows: In December 1967, the sample was dissolved at a concentration of approximately 75 mg per ml, in double-distilled water prepared with a glass still. The solution was filtered through Millipore AP20 and SC

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membrane filters and distributed in 1-ml quantities into approximately 4 000 nonactinic glass ampoules. After freeze-drying, a vented polyethylene plug was inserted into each of the ampoules, which were further dried in vacuum over phosphorus(V) oxide for 10 days. The ampoules were then filled with pure dry nitrogen, sealed by fusion of the glass, tested for leaks, and stored at −20°C in the dark.

No significant loss in weight was found on drying at 60°C *in vacuo* at a pressure of < 0.04 mmHg over phosphorus(V) oxide to constant weight.

The (first) International Standard for Polymyxin B

This was described by Humphrey et al. (1959). Its assigned potency was 7 874 IU/mg. The quantity of powder in each ampoule was small (approximately 20 mg) and it was therefore recommended that, for the collaborative assay, samples should be weighed with a micro or semimicro balance.

THE COLLABORATIVE ASSAY

Five laboratories in four countries agreed to participate in the collaborative assay. The names of the participants are listed in Annex 1, but throughout the report they are referred to by a number that has no connexion with the order of listing. Every laboratory was supplied with 5 ampoules, each containing 75 mg of the proposed second international standard for polymyxin B, together with 5 ampoules of the International Standard for Polymyxin B, each containing approximately 20 mg.

The participating laboratories were asked to assay the proposed second international standard against the first. They were informed that any recognized method of assay would be acceptable and that each assay should contain sufficient information to provide an estimate of the potency of the proposed second standard in terms of the International Standard, as well as evidence of validity and fiducial limits for that assay from its own internal evidence. It was hoped that the results of at least 4 assays, using at least 2 independent sets of weighings and dilutions, would be provided by each laboratory.

The participants were reminded that the preparations should not be dried before use. The material in the ampoules of the proposed second international standard was found to take up moisture at the rate of approximately 0.5% w/w per 5 min when exposed in an open weighing bottle at a relative humidity of 36%.

It had been hoped that some of the participating laboratories might use chemical or physical methods for the evaluation of polymyxin B, but none of them did.

Table 1. Details of assay methods used in different laboratories

Laboratory No.	Test organism	Type of assay	Dose levels of each preparation (IU/ml)	Structure of assay	No. of assays performed	Temperature of incubation
1	*Bordetella bronchiseptica* (ATCC 4617)	diffusion, plates	8.0; 10.0; 12.5	3 + 3; 1 of each concentration of each preparation on a plate; 9 plates per assay	6	37°C
2	*B. bronchiseptica* (Wellcome CN385)	diffusion, large plates	150; 300; 600	3 + 3; 4 replicates of each concentration of each preparation; 1 plate per assay	78	37°C
3	*B. bronchiseptica* (NCTC 8344)	diffusion, Petri dishes	500; 1 000; 2 000	3 + 3; 1 of each concentration of each preparation on a dish; approx. 20 dishes per assay	8	37°C
4	*B. bronchiseptica* (ATCC 4617)	diffusion, Petri dishes	25; 50; 100	3 + 3; 3 replicates of each concentration of each preparation on a dish; 30 dishes per assay	2	
5	*B. bronchiseptica* (NCTC 8344)	diffusion, large plates	40.0; 89.4; 200.0	3 + 3; 2 replicates of each concentration of one preparation and 1 of each concentration of the other preparations arranged in a 9 × 9 Latin square design; 1 plate per assay	3	37°C
			40; 200	2 + 2; 2 replicates of each concentration of each preparation arranged in an 8 × 8 Latin square design; 1 plate per assay	24	

RESULTS

The 5 laboratories performed a total of 121 assays, all by the diffusion method. Table 1 gives details of the assays performed and indicates the methods used.

STATISTICAL ANALYSIS

The conventional statistical method for parallel line assays was used for the analysis of the experimental results. The diameter of the zone of inhibition of growth was taken as the response metameter and was analysed in relation to the logarithm of the dose. The potency estimate of the proposed second standard and its precision were determined for each individual assay, and the significance of departures from parallelism and linearity of dose–response regression lines was statistically tested.

The homogeneity of the potency estimates was studied through the χ^2-test, according to the method of Humphrey et al. (1953).

When the results of a particular laboratory did not show significant heterogeneity, a weighted average logarithmic potency was computed by weighting each potency value with the reciprocal variance based on the internal evidence of the assay. The variance of this average potency was then simply the reciprocal of the total of the individual weights.

For laboratories that obtained statistically heterogeneous results, the between-assay variance was computed and the weight of each logarithmic potency value was redetermined by taking the reciprocal of the total variance—i.e., including both within-assay and between-assay variances according to the method described by Bliss (1952). The new weights were applied to the individual potencies in computing the average logarithmic potency, and its variance was taken as the reciprocal of the sum of the new individual weights.

The same method was used for computing the combined weighted log potency estimate from the laboratory average log potencies. The corresponding unweighted average estimate was also established for the purpose of comparison.

The results of the statistical tests of deviation from parallelism and linearity are summarized in Table 2. This shows that the number of assays showing a significant departure from parallelism at the 5% probability level is very small. As for departure from linearity, 12 of the 97 three-point assays carried out were nonlinear. It can be seen from the last two columns of Table 2 that the majority were concave lines.

Log potency estimates obtained by repeated assay within a single laboratory were heterogeneous in the case of 3 out of the 5 laboratories.

Laboratory 1

The 6 potency values obtained in this laboratory were homogeneous. However, the choice of doses and dose intervals was such that for several assays there was no marked difference between the responses at the low and medium doses and between those at the medium and high doses. As a consequence, 3 of the assays were statistically invalid, showing a significant departure from linearity at the 1% probability level.

Laboratory 2

Significant heterogeneity at the 1% probability level was observed among the individual potencies obtained by this laboratory, which varied from

Table 2. Significant departure [a] from parallelism and linearity—summary results

Laboratory No.	No. of assays	No. of non-parallel assays	No. of 3-point assays	No. of nonlinear assays [b]	Sign for sum of quadratics	
					−(convex)	+(concave)
1	6	1	6	3	0	3
2	78	3	78	4	1	3
3	8	3	8	2	0	2
4	2	0	2	2	0	2
5	27	4	3	1	0	1
total	121	11	97	12	1	11

[a] At the 5 % probability level of significance.
[b] With significant sum of squares for "quadratics".

Table 3. Summary results of assays of proposed second international standard for polymyxin B

Laboratory (i)	No. of assays (ii)	Homogeneity within laboratory (iii)	Weighted geometric average potency (IU/mg) (iv)	Weighted average log potency (v)	Total within-laboratory weight (vi)	Within-laboratory variance of average log potency (vii)	Total laboratory variance [a] (viii)	Total adjusted laboratory weight (ix)
1	6	homogeneous	7 902.9	3.89779	19 661.9	0.000 050 860	0.001 157 607	863.9
2	78	heterogeneous	9 429.7	3.97450 [b]	41 844.3 [c]	0.000 023 898	0.001 130 645	884.5
3	8	heterogeneous	9 136.8	3.96079 [b]	26 951.2 [c]	0.000 037 104	0.001 143 851	874.2
4	2	homogeneous	8 202.9	3.91397	8 106.5	0.000 123 358	0.001 230 105	812.9
5	27	heterogeneous	8 115.7	3.90933 [b]	29 307.9 [c]	0.000 034 120	0.001 140 867	876.5
total	121				125 871.8			4 312.0

[a] Including between-laboratory variance (0.001 106 747).
[b] Weighted with weights adjusted for within-laboratory heterogeneity.
[c] Total of adjusted weights.

9 336.2 IU/mg to 11 911.6 IU/mg. Possible sources of heterogeneity were examined, and it was seen that the greatest contribution to the total χ^2 for heterogeneity arose from variations between " ampoules/weighings " and between " days ".

Laboratory 3

There was significant heterogeneity at the 1 % probability level between the potencies of the 8 assays for this laboratory. This heterogeneity was traced to the potencies obtained from one set of " ampoules/weighings " which yielded low potency estimates.

Laboratory 4

This laboratory did 8 assays. However, only 2 were parallel line assays that could be tested for validity. The latter were found to be statistically invalid because of departure from linearity. Nevertheless, the potencies were homogeneous.

Laboratory 5

There was significant heterogeneity at the 1 % probability level of the 27 potency values obtained in this laboratory. It was traced to the potencies obtained on 1 out of the 5 assay days from one set of " ampoules/weighings ".

The final laboratory results are summarized in Table 3. The average potency values range from 7 902.9 in Laboratory 1 to 9 429.7 in Laboratory 2. The χ^2 for between-laboratory homogeneity was much greater than the critical value at the 1 % probability level for 4 degrees of freedom.

It was necessary, therefore, to take into account the additional variance component—the between-laboratory variance—in order to estimate the weighted average potency for all laboratories and its precision. The last two columns of Table 3 give the total variance and the corresponding adjusted weight for each laboratory.

The combined weight log potency estimate was computed by applying to each laboratory log potency given in column (v) of Table 3 the corresponding weight shown in column (ix). The variance of this general average log potency was estimated as the reciprocal value of the sum of the total adjusted laboratory weights (1/4 312=0.00023191).

The resulting combined potency of the proposed second international standard for polymyxin B was 8 544.6 IU/mg with a 95% confidence interval of 7 977.0–9 152.5 IU/mg.

For the purpose of comparison, the general unweighted geometric mean potency was calculated separately for all the 121 assays and for the valid assays only (i.e., excluding the assays with a statistically significant departure from parallelism and/or linearity).

The results were as follows.

Coverage	*No. of assays*	*Unweighted geometric mean potency (IU/mg)*	*95% confidence interval (IU/mg)*
All assays	121	9 015.0	8 838.4–9 195.1
Valid assays	100	9 092.0	8 898.4–9 289.7

Table 4. Summary results of assays of proposed second international standard for polymyxin B (based on 70 assays selected to produce homogeneity within laboratories)

Laboratory (i)	No. of assays (ii)	Homogeneity within laboratory (iii)	Weighted geometric average potency (IU/mg) (iv)	Weighted average log potency (v)	Total within-laboratory weight (vi)	Within-laboratory variance of average log potency (vii)	Total laboratory variance [a] (viii)	Total adjusted laboratory weight (ix)
1	6	homogeneous	7 902.9	3.89779	19 661.9	0.000 050 860	0.000 745 434	1 341.5
2	36	homogeneous	8 650.4	3.93704	214 224.1	0.000 004 668	0.000 699 252	1 430.1
3	6	homogeneous	9 319.7	3.96940	84 579.3	0.000 011 823	0.000 706 414	1 415.6
4	2	homogeneous	8 202.9	3.91397	8 106.5	0.000 123 358	0.000 817 929	1 222.6
5	20	homogeneous	8 368.0	3.92262	225 188.3	0.000 004 441	0.000 699 007	1 430.6
total	70				551 760.1			6 840.4

[a] Including between-laboratory variance (0.000 694 578).

Table 4 shows the effect of excluding from the final analysis the assays from Laboratories 2, 3, and 5 that appeared to be the most discrepant and the exclusion of which left a homogeneous group of assays within each laboratory. In this way a combined weighted mean potency of 8 489.8 IU/mg with a 95% confidence interval of 8 039.0–8 966.0 IU/mg was obtained. The results from Laboratory 3 form two groups: one group consisting of 2 assays, which was rejected in the above-mentioned calculation, and another group of 6 assays. When the latter group was rejected instead of the former, a combined weighted mean potency of 8 362.2 IU/mg was obtained with confidence limits of 8 099.2–8 633.7 IU/mg. A value lying between the two weighted mean potency values was considered to be the best estimate of the potency of the second international standard for polymyxin that the study could provide.

DISCUSSION

The very great heterogeneity of the potency values obtained in this study, both within and between laboratories, may stem in part from differences in composition between the first international standard, which was manufactured prior to 1954, and the second standard, which is representative of current production. This difference may have resulted from variations in the content of polymyxins B_1 and B_2, but unfortunately insufficient material was available to allow this analysis to be carried out on the first international standard. Apart from this possible source of variation in assay, a number of laboratories found great difficulty in opening ampoules of the first international standard without contaminating the contents with glass fragments. This, together with the small amount of powder in each ampoule, the difficulty of removing it from the ampoules owing to static charge, and its rapid uptake of moisture during manipulation, made it very difficult to obtain samples weighed with great precision.

Since supplies of the first international standard were exhausted, the National Institute for Medical Research, in accordance with the authorization of the WHO Expert Committee on Biological Standardization (1969), established the material that had been examined as the second International Standard for Polymyxin B and suggested that the International Unit of polymyxin B be defined as the activity contained in 0.000119 mg of the preparation—a value corresponding to a potency lying within the range suggested by the collaborative assay, i.e., 8 362.2 to 8 489.8 IU/mg.

ESTABLISHMENT OF THE INTERNATIONAL STANDARD AND DEFINITION OF THE INTERNATIONAL UNIT

The WHO Expert Committee on Biological Standardization (1970) considered the data that had been obtained in the collaborative assay and decided that, since it was unlikely that a more accurate estimate of potency could be made and since there was an urgent need for the unit to be defined, this should be done on the basis of the available results. The value suggested was acceptable to the collaborating laboratories, and the Expert Committee therefore defined the International Unit of polymyxin B as the activity contained in 0.000119 mg of the second International Standard for Polymyxin B. This corresponds to a potency of 8 403 IU/mg.

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RÉSUMÉ

DEUXIÈME ÉTALON INTERNATIONAL DE POLYMYXINE B

Les stocks du premier étalon international de polymyxine B, constitué en 1955, étant épuisés, le National Institute for Medical Research, de Londres, s'est procuré un échantillon de 400 g de sulfate de polymyxine B pouvant servir à constituer un deuxième étalon. Dissous dans l'eau, réparti en ampoules et lyophilisé, ce matériel a été distribué à cinq laboratoires de quatre pays pour être soumis à un titrage comparatif avec le premier étalon international.

On a pratiqué au total 121 épreuves par diffusion en utilisant comme organisme d'épreuve *Bordetella bronchiseptica*. Elles ont donné des résultats très hétérogènes, les estimations de l'activité variant à la fois dans un même laboratoire et entre les laboratoires. La principale raison de ces écarts semble être les difficultés rencontrées lors de la manipulation des ampoules contenant le premier étalon international.

Après exclusion des résultats les plus discordants, on a obtenu en combinant les données fournies par les laboratoires deux valeurs moyennes d'activité: 8489,8 UI/mg, avec des limites de confiance 95% de 8039,0 à 8966,0 UI/mg, et 8362,2 UI/mg, avec des limites de confiance 95% de 8099,2 à 8633,7 UI/mg. Estimant qu'il était peu probable qu'on puisse évaluer l'activité du deuxième étalon avec davantage de précision en procédant à de nouvelles épreuves, les participants au titrage comparatif ont constitué le matériel en deuxième étalon international de polymyxine B et proposé que l'unité internationale de polymyxine B soit définie comme l'activité de 0,000119 mg du deuxième étalon international. Cette valeur, correspondant à une activité de 8403 UI/mg, a été acceptée par le Comité OMS d'experts de la Standardisation biologique (1970) lorsqu'il a défini l'unité internationale de polymyxine B par rapport au deuxième étalon international.

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Annex

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