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INTERNATIONAL STANDARD FOR ERYTHROMYCIN

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SYNOPSIS

A batch of highly purified erythromycin A has been examined by 9 laboratories in 6 different countries, and has been assayed against the erythromycin standard of the Food and Drug Administration of of the US Department of Health, Education, and Welfare. The material examined has been established as the International Standard for Erythromycin, and the International Unit of Erythromycin is defined as the activity contained in 0.001053 mg of the International Standard. The International Unit is, for practical purposes, equivalent to one μg of pure erythromycin base.

At the eighth session of the WHO Expert Committee on Biological Standardization, held at Geneva in 1954,[1] the Department of Biological Standards of the National Institute for Medical Research, London, was authorized to obtain a preparation of erythromycin suitable for an International Standard, and to proceed with its characterization and assay. At the ninth session, in 1955,[2] it was reported that a quantity of highly purified erythromycin and a suitable reference preparation had been obtained. At the tenth session, in 1956,[3] it was reported that an international collaborative assay had been carried out. The Committee authorized the National Institute for Medical Research to establish the material examined as the International Standard for Erythromycin and to assign to it a unitage based on the results of the collaborative assay, with the agreement of the participants.

Proposed International Standard

The material, consisting of 400 g of a single batch of highly purified erythromycin (" Ilotycin ", lot 64937), was obtained through the generosity of Messrs Eli Lilly & Co., USA. It was received at the National Institute for Medical Research in a single sealed container, which was

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1955, **96**, 12
[2] *Wld Hlth Org. techn. Rep. Ser.*, 1956, **108**, 13
[3] *Wld Hlth Org. techn. Rep. Ser.*, 1957, **127**, 13

stored at — 10°C for 9 weeks, when the contents were distributed in approximately 200-mg quantities into hard glass ampoules. The ampoules were stored *in vacuo* over P_2O_5 at room temperature for 12 days, during which the P_2O_5 was changed four times. They were then filled with pure dry N_2 and sealed. Moisture content (loss in weight on drying for 3 hours at 60°C and 0.1 mm Hg) on 5 ampoules taken at random was 0.045%. Additional tests showed that the material in the ampoules picked up moisture only slowly when exposed to the atmosphere, and there was therefore no reason to suspect any heterogeneity due to variable uptake of moisture during the sealing of the ampoules. The ampoules have been tested for leaks since sealing. They are stored at −10°C.

Reference Preparation

The Reference Preparation was kindly provided by Dr H. Welch of the Food and Drug Administration (FDA) of the United States Department of Health, Education, and Welfare. It consisted of 5 g of a crystalline preparation of erythromycin which, at the time of its receipt in June 1955, was under consideration for use as the standard preparation of the FDA and of the US Pharmacopeia. The material was stored for 4 months at 4°C in a sealed container over silica gel, after which it was distributed into glass ampoules, which were filled with dry N_2 and sealed, without further drying. The properties of the Reference Preparation (information supplied by Dr D. Grove, of the FDA) were as follows:

solubility analysis	= 99 + % single component
x-ray diffraction	= essentially no impurities
apparent molecular weight	= 750
melting point	= indefinite in range 130-141°C
moisture (Karl Fischer)	= 3.9%

(The moisture content found at the National Institute for Medical Research was 4.3% and by Messrs Eli Lilly & Co. 4.5%.)

The Reference Preparation is considered by the FDA to consist of 98% erythromycin base, after drying for 4 hours at 70°C and a pressure of 10 mm Hg—the material as distributed into ampoules being therefore 93.8% erythromycin.

Chromatographic examination of the Proposed International Standard and the Reference Preparation was undertaken at the National Institute for Medical Research. They were examined by paper chromatography using three different solvent systems, and there was no evidence of significant amounts of more than one component possessing biological activity. The infra-red spectra of the two materials show only very minor differences. As a further test for the presence of erythromycin B, the biological activity of both preparations was examined after holding in 0.4% solution at

pH 1.5 for 40 min., followed by restoration to pH 7.0. The amount of biological activity which was stable under these conditions was only 3.4% and 2.9% for the Proposed International Standard and the Reference Preparation respectively.

Collaborative Assay

Nine laboratories in 6 different countries took part in a collaborative assay of the Proposed International Standard against the Reference Preparation. A full list of these laboratories is given in the Annex, but throughout this report they are referred to by number only. In all, 249 assays were carried out—an assay being defined as the amount of information that will provide an independent estimate of potency. Of these, 197 were biological assays, and, in the remaining 52, spectrophotometric methods were used. Table I lists the number of assays performed, and the method and organism used, by each laboratory.

Statistical Analysis

It has been the usual practice of the Department of Biological Standards in collaborative assays to carry out a complete analysis of variance on all

TABLE I. NUMBER AND TYPE OF ASSAY USED BY PARTICIPATING LABORATORIES

Laboratory No.	Type of assay	Organism	Number of assays
1	Plate	*Micrococcus pyogenes*	2
	Spectrophotometric		2
2	Plate	*Sarcina lutea*	6
	Plate	*Bacillus subtilis*	6
3	Plate	*Bacillus pumilis*	8
4	Plate	*Staphylococcus aureus*	8
5	Plate	*Sarcina lutea*	5
	Turbidimetric	*Micrococcus aureus*	5
6	Turbidimetric	*Staphylococcus aureus*	18
	Spectrophotometric		10
7	Plate	*Sarcina lutea*	21
	Plate	*Bacterium mycoides*	23
8	Plate	*Bacillus subtilis*	31
	Spectrophotometric		16
9	Plate	*Bacillus subtilis*	64
	Spectrophotometric		24

results presented in a form susceptible to such analysis, and so to give a statistical weight to each assay which can be used for the computation of an over-all weighted mean potency. This procedure becomes very time-consuming when a large number of assays are involved, and it has furthermore been noted in previous collaborative assays of antibiotics that difficulties arise in interpreting the statistical results, owing to the extreme precision of such assays. Because the residual error terms are often very small, the slightest deviations (of the log-dose response lines) from linearity or parallelism become statistically significant, and, according to usual statistical practice, should lead to the rejection as invalid of the assay in which they occur. This point has been discussed at length in the memorandum on the International Standard for Aureomycin.[1]

We have considered various alternative methods of analysis, and these are discussed below.

One possibility is to use some metameter other than the response that is actually measured, e.g., the square of the zone diameter instead of the diameter, in a plate assay. This is sometimes successful in giving linear log-dose response lines, and sometimes not, according to the degree of curvature of the original lines.

Another possibility is to consider that the estimate of potency is valid but the error variance is underestimated. By this method the error term is increased to a point at which the component for curvature is no longer significant, thereby decreasing the weight of the assay but leaving the single estimate of potency unaltered.

Whenever a group of assays have been analysed by more than one method it has been found that the over-all estimates of potency have been almost identical, although the weights associated with those estimates have varied.

A detailed investigation of the eight large plate assays from Laboratory No. 3 has been made in order to illustrate these points. Using the zone diameter as response, analysis of variance gives a significant curvature at the 1% level in six of the eight assays. If this curvature is ignored a weighted mean potency of 977 μg equiv./mg is obtained for the eight plates. Adjusting the weight of each assay, according to the degree of curvature, the estimate of potency becomes 973 μg equiv./mg.

Another two estimates of 977 μg equiv./mg and 983 μg equiv./mg are made by following the same procedure but using the squared diameter as response. When this metameter is used, four of the log-dose response lines exhibit a significant curvature, these being the ones that were closest to linearity when the diameter was used.

Finally, consideration has been given to using the " direct " method of analysis, in which the internal evidence of each assay is completely ignored,

[1] *Bull. Wld Hlth Org.* 1953, 9, 851

and the calculation made directly from the normal distribution of the eight individual log potencies. The estimate of potency is now the antilog of $\bar{M}$ (the mean log potency) and this value is weighted by the reciprocal of V_M (the variance of the mean log potency).

The results of these various methods of analysis are summarized in Table II.

TABLE II. MEAN POTENCIES OBTAINED BY ANALYSING THE ASSAYS FROM LABORATORY No. 3 BY DIFFERENT METHODS

Method of analysis	Potency (μg equiv./mg)	Weight
Diameters (ignoring curvature)	977	171 137
Squared diameter (ignoring curvature)	977	163 994
Diameter (reducing weight)	973	90 655
Squared diameter (reducing weight)	983	87 200
Direct method	981	171 250

All calculations of potency are based on the assumption that the Reference Preparation contained 93.8% of erythromycin and are quoted for convenience in terms of microgram-equivalents of erythromycin per milligram (μg equiv./mg).

It will be seen that the most extreme estimates of potency differ by only 1% and that the weight obtained by the direct method of analysis is almost identical with the fully weighted estimates based on the internal evidence of each assay. Even the smallest weight leads to limits of error of only ± 1.5% of the potency.

It would appear from this that use of the direct method of analysis in antibiotic assays leads to conclusions not significantly different from those arrived at by far more elaborate methods. Moreover in any collaborative assay there are always a number of assays submitted which have been designed in such a way that, apart from the direct method, no analysis is possible. Some participating laboratories, furthermore, use a (2 + 2) dose design, which does not permit detection of curvature, if it exists, and it seems questionable to give these assays their full weight if other assays of a more elaborate design are to be reduced in weight. Of the biological assays submitted for erythromycin, 48% are of this design, while another 24% could only be interpreted by the direct method. A complete analysis, including the test for curvature, is only possible for the remaining 28%.

For these reasons the estimates of potency quoted in this report have all been made by the direct method.

Results

Following the usual practice, only the biological assays have been used in the determination of the over-all potency. The mean estimates of potency, and associated weights, obtained by all biological methods in individual laboratories are shown in Table III.

Although the twelve estimates of potency in Table III appear to be fairly consistent the χ^2 for homogeneity is highly significant ($\chi^2=80.49$, $P<0.001$). This is another effect of high weighting which is commonly encountered in antibiotic assays.

TABLE III. MEAN POTENCIES OBTAINED BY DIFFERENT BIOLOGICAL METHODS IN DIFFERENT LABORATORIES

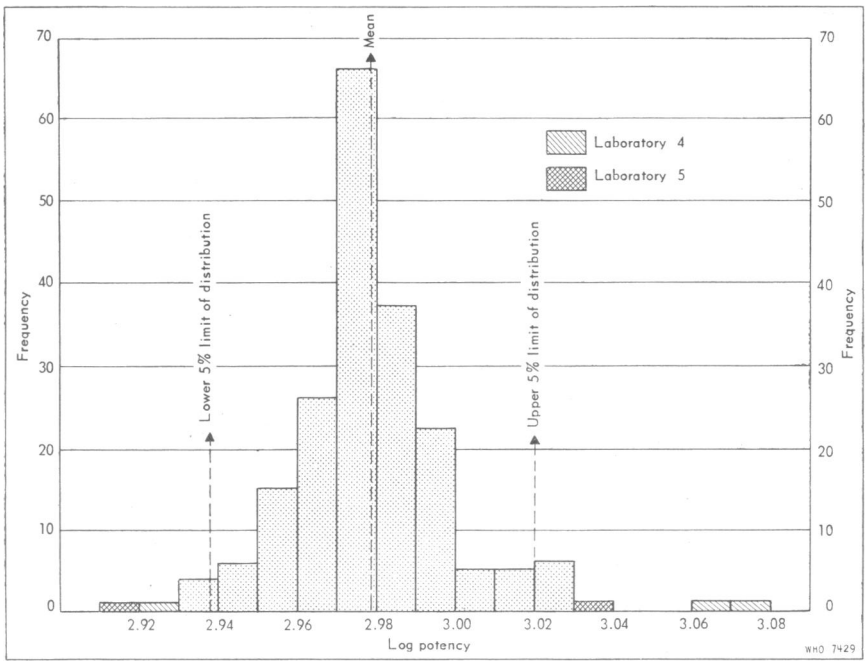
Laboratory No.	Number of assays	Potency (μg equiv./mg)	Weight
1	2	968	13 760
2	6	920	109 885
	6	940	1 299 157
3	8	981	171 250
4	8	1 027	3 387
5	5	960	4 834
	5	944	2 317
6	18	953	63 354
7	21	950	576 033
	23	949	1 956 318
8	31	955	45 543
9	64	946	251 135
	Total: 197		Total: 4 496 973

Disregarding heterogeneity the weighted mean potency for all laboratories is 947 μg equiv./mg with 5% limits of error 945 to 949 μg equiv./mg, i.e., 99.8% to 100.2% of the potency.

The direct method of analysis can be applied to the distribution of the 197 individual log potencies (see figure below). It will be seen that the latter are normally distributed about a mean of 2.979, giving an over-all mean potency of 952 μg equiv./mg with 95% of all assays falling within ± 10% of the mean.

The long tails of the distribution are not due to any material differences in the values obtained by different laboratories, but to the variability of the results from Laboratories Nos. 4 and 5.

FREQUENCY DISTRIBUTION OF LOG POTENCIES DERIVED FROM 197 BIOLOGICAL ASSAYS



The limits of the mean potency are 945 to 958 μg equiv./mg, i.e., 99.3% to 100.7% of the mean. Limits calculated in this way reflect the variation between individual estimates of potency due to all possible causes. That the variation between laboratories is not serious can be seen from the fact that these limits differ by only 0.5% from those which were estimated by weighting each laboratory separately.

The results obtained in the spectrophotometric assays are listed in Table IV and agree reasonably well with those obtained by bio-assay.

TABLE IV. POTENCIES OBTAINED BY SPECTROPHOTOMETRIC METHOD

Laboratory No.	Number of assays	Potency (μg equiv./mg)
1	2	980
6	10	970
8	16	977
9	24	954
	Total : 52	

The participants in the international collaborative assay are agreed that the biological potency of the material examined be taken as 950 μg equiv./mg, in terms of the Reference Preparation. In accordance with the decision of the WHO Expert Committee on Biological Standardization, the International Unit of Erythromycin is defined as nearly as possible equivalent to the activity of 1 μg of the pure substance. The potency of the material is, therefore, 950 International Units per mg and *the International Unit of Erythromycin is defined as the activity contained in 0.001053 mg of the International Standard for Erythromycin.*

Annex

PARTICIPANTS IN THE COLLABORATIVE ASSAY OF THE PROPOSED INTERNATIONAL STANDARD FOR ERYTHROMYCIN

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RÉSUMÉ

Un lot d'érythromycine A hautement purifiée a été examiné par 9 laboratoires, dans 6 pays, et comparé à l'érythromycine étalon de la Food and Drug Administration des Etats-Unis d'Amérique. Ce matériel constitue désormais l'Etalon international d'Erythromycine et l'Unité internationale d'Erythromycine a été définie comme correspondant à l'activité exercée par 0,001053 mg de l'Etalon international. Pour des raisons pratiques, l'Unité internationale a été définie de façon qu'elle corresponde à 1 μg d'érythromycine base pure.