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# The International Reference Preparation of Hygromycin B *

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*The Central Veterinary Laboratory, Weybridge, England, was requested by the WHO Expert Committee on Biological Standardization to obtain suitable material for an international reference preparation of hygromycin B and to arrange a collaborative assay. A batch of 150 g of a highly purified hygromycin B was assayed in eleven laboratories in nine countries against the hygromycin B standard of a commercial manufacturer. On the basis of the results obtained, the material has been established as the International Reference Preparation of Hygromycin B and the International Unit for Hygromycin B has been defined as the activity contained in 0.0008928 mg of the International Reference Preparation of Hygromycin B.*

The WHO Expert Committee on Biological Standardization (1964a) asked the Central Veterinary Laboratory, Weybridge, to obtain a quantity of hygromycin B suitable for use as an international reference preparation and to arrange a collaborative assay.

Eli Lilly & Co., Indianapolis, Ind., USA, generously donated 150 g of a highly purified preparation of hygromycin B. The purity of the preparation had been confirmed by paper chromatographic analysis and its identity with the manufacturer's house standard demonstrated by infrared and nuclear magnetic resonance spectroscopy. Its potency was stated to be 1106 units/mg.

The preparation was distributed into ampoules with stainless steel measures so that each ampoule contained about 40 mg. The ampoules were dried over phosphorus pentoxide for four weeks, filled with dry nitrogen and sealed. They were then stored at −20°C.

## THE INTERNATIONAL COLLABORATIVE ASSAY

Eleven laboratories in nine countries took part in the collaborative assay. They are listed in Annex 1 and referred to in the text and tables by arbitrary numbers that do not necessarily correspond to the order of listing in Annex 1.

A quantity of Eli Lilly's house standard (H), to which the manufacturer had assigned a potency of 1106 units/mg, was obtained. Samples of this, together with ampoules of the proposed reference preparation (RPH), were sent to the collaborators.

Each laboratory was asked to perform by its own methods a series of assays designed to compare the potencies of the two preparations and was also asked to include, where possible, a locally available preparation of hygromycin B. Laboratories were asked to design their assays so that three or more dilutions of each preparation were tested simultaneously and to incorporate several independent weighings of each preparation. Table 1 gives the principal features of the assay methods used.

## RESULTS AND STATISTICAL ANALYSIS

Analysis of assays from all the laboratories except Laboratories 8 and 9 has been carried out by the normal method relating zone diameter to log dose. All assays of preparation RPH against preparation H have been considered valid, as there were no highly significant deviations from parallelism of the log-dose–response lines, although three assays showed deviations that were just significant ($P < 0.05$). In some cases, however, there was significant curvature of the lines, and for Laboratories 1 and 2 additional analyses were carried out using the square of the zone diameter (Humphrey et al., 1959). This tended to improve the linearity but, as the potency estimates remained almost unchanged, this method of analysis was not extended to the data from other laboratories. At Laboratory 11 the combined curvature was significant in all but one of the assays, but this was attributed to the high precision found.

The results of all the tests are given in Annex 2.

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TABLE 1. ASSAY METHODS

| Laboratory No. | Type of assay | Test organism | Statistical design [a] |
|---|---|---|---|
| 1 | Plate | *B. subtilis*, NCTC 8236 | 6×6, RLS |
| 2 | Plate | *B. subtilis*, NCTC 8236 | 8×8, RLS |
| 3 | Plate | *B. subtilis*, ATCC 6633 | 3 dilutions, 8 replicates/assay |
| 4 | Plate | *B. subtilis*, local strain | 6×6, LS |
| 5 | Plate | *B. subtilis*, ATCC 6633 | 3 dilutions, 9-10 replicates/assay |
| 6 | Plate | *B. subtilis*, ATCC 6633 | 6×6, LS |
| 7 | Plate | *B. subtilis*, ATCC 6633 | 4 dilutions, 4 replicates/assay |
| 8 | Turbidimetric | *B. subtilis*, local strain | 12 dilutions, 5 replicates/assay |
| 9 | Plate | *B. subtilis*, local strain | 1 dilution, 3 replicates/assay |
| 10 | Plate | *B. subtilis*, PIC 219 | 3 dilutions, 7 replicates/assay |
| 11 | Plate | *B. subtilis*, ATCC 6633 | 6×6, RLS |

[a] LS: Latin Square; RLS: Randomized Latin Square.

Potency ratios for preparation RPH (test) relative to preparation H (standard), weight for weight, are shown, with 95% confidence intervals in parentheses, followed by the statistical weight (the reciprocal of the variance of the log potency), the validity and finally, where more than one test was carried out on a single pair of weighings, the combined potency and the confidence interval for the assay. Five of the collaborating laboratories performed more than one test on independent pairs of weighings, the number ranging from two to four. Validity tests indicate the significance of the curvature of the log-dose–response lines, where this occurred, and values of $\chi^2$ the homogeneity of potency estimates, both between and within ampoules.

Assays from Laboratory 8, which employed a turbidimetric method using 12 doses of both test and standard preparations, were analysed by direct comparison of the linear portion of the log-dose–response curves, the weight used for each assay being calculated from the combined variance of the two regression lines. A standard-curve method was used at Laboratory 9, and no estimate of the error for separate tests was possible. The statistical weights for all tests for the two assays on each pair of ampoules have therefore been calculated directly from the reciprocal of the variance of the geometric mean potency ratios.

At Laboratory 3, preparations H and RPH were assayed separately against a local preparation (LPH) and potency ratios have been calculated indirectly from the estimates for each set of weighings.

Laboratory 11 assayed each ampoule of preparation RPH against each ampoule of H (9 assays), but unfortunately the three assays using ampoule RPH3 had to be excluded as no estimate of error was available.

*House standards*

Three other laboratories, in addition to Laboratory 3, included local preparations. Laboratory 6 performed assays of its own house standard (LPH) against preparation RPH, using the same dilutions of the latter preparation as were used in the assays against preparation H. Laboratory 10 assayed its local preparation against each ampoule of RPH used in the main assay, and Laboratory 11 carried out one assay on each of two different local preparations, also comparing them with RPH.

The house-standard preparations included by Laboratories 3 and 6 were Eli Lilly preparations with a stated potency of 1065 and 1000 units/mg, respectively. For Laboratory 3 the potency ratio of LPH (test) and H (standard) in direct assays was 0.8744, the dilutions used having been equated on the basis that LPH contained 1065 units/mg and H 1000 units/mg. The relative potency, weight for weight, is thus 0.9312, giving a value of 1030 units/mg (1014-1047 units/mg) for LPH, as H is stated to have a potency of 1106 units/mg. The potency of LPH relative to H, weight for weight, obtained indirectly from the combined assay results for Laboratory 6, is 0.8853. i.e., the local preparation has an estimated potency of 979 units/mg, assuming a potency of 1106 units/mg for Preparation H. The estimated potencies of these two house standards therefore differ by less than 4% from the potencies assigned to them.

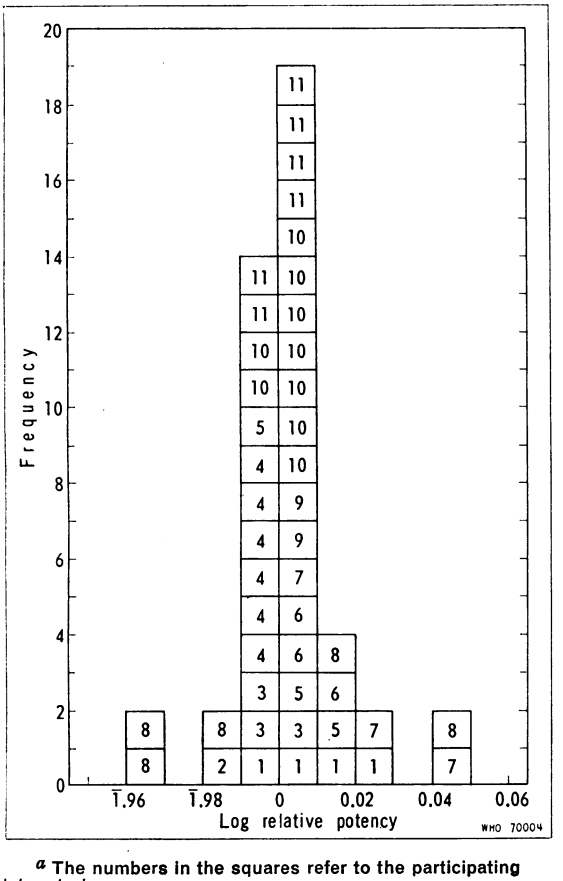
The potency of the local preparation used by Laboratory 10, again calculated indirectly, is 0.9817 relative to that of preparation H, corresponding to

1086 units/mg, and for the two preparations used at Laboratory 11 the estimated potencies are 0.8623 and 0.9638, corresponding to 954 and 1066 units/mg, respectively. There was a highly significant deviation from parallelism with RPH in the second of these, but this is again attributable to the high weighting. None of the other local preparations included by the four laboratories differed significantly in slope from H or RPH.

*Over-all potency estimate*

Table 2 shows the weighted geometric mean relative potencies for all assays (independent pairs of weighings) and their statistical weights. The frequency distribution of the 45 log potencies is shown in the figure.

FREQUENCY DISTRIBUTION OF LOG RELATIVE POTENCIES OF THE INTERNATIONAL REFERENCE PREPARATION OF HYGROMYCIN B OBTAINED IN DIFFERENT LABORATORIES (45 ASSAYS) [a]




[a] The numbers in the squares refer to the participating laboratories.

TABLE 2
WEIGHTED MEAN RELATIVE POTENCIES FOR 45 INDEPENDENT ASSAYS

| Laboratory No. | Relative potency | Weight |
|---|---|---|
| 1 | 1.052 | 22 109 |
| | 1.006 | 40 483 |
| | 1.024 | 54 436 |
| | 0.998 | 36 674 |
| 2 | 0.963 | 1 568 |
| 3 [a] | 1.008 | 26 344 |
| | 0.999 | 29 608 |
| | 0.993 | 26 845 |
| 4 | 0.997 | 7 263 |
| | 0.996 | 8 837 |
| | 0.994 | 17 192 |
| | 0.998 | 7 794 |
| | 0.993 | 8 081 |
| | 0.995 | 8 126 |
| 5 | 0.987 | 50 861 |
| | 1.045 | 40 222 |
| | 1.014 | 47 768 |
| 6 | 1.010 | 19 519 |
| | 1.010 | 1 449 |
| | 1.040 | 1 685 |
| 7 | 1.017 | 2 610 |
| | 1.064 | 4 959 |
| | 1.099 | 9 587 |
| 8 | 0.917 | 13 240 |
| | 0.914 | 1 559 |
| | 0.955 | 1 675 |
| | 1.037 | 4 032 |
| | 1.102 | 9 911 |
| 9 [a] | 1.006 | 2 895 |
| | 1.001 | 5 823 |
| 10 | 1.017 | 14 182 |
| | 1.000 | 12 845 |
| | 0.993 | 16 072 |
| | 1.018 | 15 412 |
| | 1.014 | 18 120 |
| | 1.014 | 11 142 |
| | 1.007 | 10 226 |
| | 1.007 | 17 152 |
| | 1.011 | 17 435 |
| 11 | 1.006 | 22 962 |
| | 1.000 | 39 904 |
| | 1.013 | 59 400 |
| | 1.000 | 39 904 |
| | 1.006 | 23 895 |
| | 1.018 | 26 918 |

[a] Indirect estimates.

The weighted geometric mean potency ratios for assays at each laboratory are given in Table 3, with their total weights and values of $\chi^2$ for heterogeneity. As there was a highly significant difference between log potencies at Laboratory 8, the unweighted mean log potency and its weight (the reciprocal of the variance of the mean) are given in parentheses.

The values of $\chi^2$ for the nine laboratories that employed plate methods and compared preparations H and RPH directly were:

Within laboratories $\chi^2 = 28.42$ (28 DF) $0.50 > P > 0.30$
Between laboratories $\chi^2 = 17.89$ ( 8 DF) $0.05 > P > 0.02$

The difference between laboratories was only just significant; the over-all weighted geometric mean potency was calculated (Humphrey et al., 1953) and was found to be 1.0113, with a 95% confidence interval of 1.0059–1.0164 and a weight of 745 510. Inclusion of Laboratory 3, which used indirect assays, gave a value of 1.0100 (1.0050–1.0150) with a weight of 828 307. If it is again assumed that preparation H has a potency of 1106 units/mg, the potency of preparation RPH is 1118 units/mg (1113–1123 units/mg) for the direct assays, and 1117 units/mg (1112–1123 units/mg) if Laboratory 3 is included.

As some laboratories carried out more than one test on a single pair of weighings, the over-all weighted potency was also estimated using the mean weight per assay instead of the total weight. However, the final potency was found to differ by less than 0.1% from the estimate based on total weights.

Calculation of the unweighted mean potency of preparation RPH directly from the normal distribution of the log potencies for each assay gave a value of 1119 units/mg (1110–1127), with a weight of 372 953, for the nine laboratories that used direct assay by plate methods, and 1118 units/mg (1110–1126 units/mg), with a weight of 425 858, when Laboratory 3 was included. These values are very close to the previous estimates. When the results from Laboratory 8, which used a turbidimetric method, are added, the estimated potency is 1114 units/mg (1103–1125 units/mg), with a weight of 210 608.

## CONCLUSION

The WHO Expert Committee on Biological Standardization (1966) noted that the proposed reference preparation (RPH) had been established, in accordance with the authorization in its seventeenth report (WHO Expert Committee on Biological

TABLE 3
SUMMARY OF RESULTS OBTAINED AT EACH LABORATORY

| Laboratory No. | No. of assays [a] | Weighted geometric mean relative potency | Weight | Homogeneity of potency estimates between independent assays | | |
|---|---|---|---|---|---|---|
| | | | | $\chi^2$ | Degrees of freedom | P |
| 1 | 4 | 1.017 | 153 702 | 8.69 | 3 | 0.05>P>0.02 |
| 2 | 1 | 0.963 | 1 568 | — | | |
| 3 | (3) | 1.000 | 82 797 | 0.53 | 2 | 0.80>P>0.70 |
| 4 | 6 | 0.995 | 57 293 | 0.03 | 5 | P>0.99 |
| 5 | 3 | 1.013 | 138 851 | 13.53 | 2 | 0.01>P>0.001 |
| 6 | 3 | 1.012 | 22 653 | 0.25 | 2 | 0.90>P>0.80 |
| 7 | 3 | 1.076 | 17 156 | 2.54 | 2 | 0.30>P>0.20 |
| 8 | 5 | 0.992 (0.983) [b] | 30 417 (787) [c] | 40.27 | 4 | P<0.001 |
| 9 | (2) | 1.002 | 8 718 | 0.02 | 1 | 0.90>P>0.80 |
| 10 | 9 | 1.009 | 132 586 | 1.58 | 8 | P>0.99 |
| 11 | 6 | 1.007 | 212 983 | 1.78 | 5 | 0.90>P>0.80 |

[a] Figures in parentheses are number of indirect comparisons (Lab. 3) or of grouped assays (Lab. 9).
[b] Value in parentheses is unweighted mean potency.
[c] Value in parentheses is weight of the unweighted mean potency that it follows.

Standardization, 1964b), as the International Reference Preparation of Hygromycin B, and, on the basis of the results of the collaborative assay, the Committee defined the International Unit for Hygromycin B as the activity contained in 0.0008928 mg of the International Reference Preparation of Hygromycin B. The International Unit for Hygromycin B is thus equivalent, within the limits of experimental error, to the unit of potency defined by Eli Lilly & Co.

## RÉSUMÉ

En 1964, le Comité OMS d'experts de la Standardisation biologique notait la nécessité de disposer d'une préparation internationale de référence d'hygromycine B et demandait au Central Veterinary Laboratory, de Weybridge, Angleterre, de se procurer du matériel approprié et d'organiser un titrage comparatif.

Un lot de 150 g d'une préparation très purifiée d'hygromycine B a pu être obtenu et a fait l'objet, dans 11 laboratoires de 9 pays, d'une étude collective visant à comparer son activité à celle de l'étalon d'hygromycine B d'une firme commerciale.

Sur la base des observations effectuées dans les différents laboratoires, et après analyse statistique des données, le matériel de référence proposé a été constitué en préparation internationale de référence d'hygromycine B. L'unité internationale de référence d'hygromycine B a été définie comme l'activité de 0,0008928 mg de la préparation internationale de référence.

## REFERENCES

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*Annex 1*

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*Annex 2*

## RESULTS OF STATISTICAL ANALYSIS OF INDIVIDUAL ASSAYS

### LABORATORY 1

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) | Weight | Validity [a] | Combined potency (and 95 % confidence interval) |
|---|---|---|---|---|---|
| 1 | H1, RPH2 | 1.023 (0.949-1.102)<br>1.062 (1.001-1.127)<br>1.064 (1.005-1.128)<br>1.046 (0.974-1.124) | 4 131<br>6 574<br>6 936<br>4 468 | | 1.052<br>(1.021-1.084) |
| 2 | H2, RPH5 | 0.995 (0.960-1.033)<br>0.992 (0.929-1.058)<br>0.995 (0.940-1.052)<br>1.041 (0.994-1.091) | 17 158<br>5 445<br>7 281<br>10 599 | S : P<0.001<br>C : P<0.05<br>C : P<0.01 | 1.006<br>(0.984-1.029) |
| 3 | H3, RPH4 | 1.034 (0.991-1.078)<br>1.033 (0.973-1.096)<br>1.013 (0.978-1.049)<br>1.027 (0.988-1.066) | 12 897<br>6 526<br>18 998<br>16 015 | C : P<0.001<br>D : P<0.05 | 1.024<br>(1.004-1.044) |
| 4 | H3, RPH4 | 0.982 (0.938-1.027)<br>1.005 (0.956-1.058)<br>1.020 (0.964-1.080)<br>0.992 (0.943-1.043) | 11 286<br>9 003<br>7 131<br>9 254 | | 0.998<br>(0.974-1.021) |

[a] C = combined curvature of dose–response curves significant.
S = separate curvature of dose–response curves significant.
D = significant departure from parallelism.

Test for Heterogeneity:

| | $x^2$ | Degrees of freedom | Significance [b] |
|---|---|---|---|
| Within ampoules | 6.21 | 12 | NS |
| Between ampoules | 5.83 | 2 | NS [c] |
| Between assays 3 and 4 | 2.86 | 1 | NS |
| Total | 14.90 | 15 | NS |

[b] NS = not significant. [c] P $\simeq$ 0.05.

*Annex 2 (continued)*

LABORATORY 2

| Assay | Ampoules | Potency ratio (and 95 % canfidence interval) [a] | Weight | Combined potency (and 95 % confidence interval) |
|---|---|---|---|---|
| 1 | H1, RPH1 | 0.906 (0.768-1.069)<br>1.024 (0.867-1.211) | 792<br>776 | 0.963<br>(0.859-1.079) |

[a] Validity: Significant departure from parallelism ($P<0.05$) in first test.

Test for Heterogeneity:

| $\chi^2$ | Degrees of freedom | Significance |
|---|---|---|
| 1.12 | 1 | Not significant |

LABORATORY 3

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) [a, b] | Weight | Combined potency (and 95 % confidence interval) |
|---|---|---|---|---|
| 1 | LPH1, H1 | 1.155 (1.103-1.210) | 10 074 | 1.166 |
| | | 1.177 (1.126-1.231) | 10 779 | (1.131-1.204) |
| | LPH1, RPH1 | 1.179 (1.136-1.225) | 15 182 | 1.176 |
| | | 1.172 (1.131-1.215) | 16 654 | (1.146-1.206) |
| 2 | LPH2, H2 | 1.117 (1.075-1.160) | 14 938 | 1.131 |
| | | 1.144 (1.105-1.184) | 17 946 | (1.104-1.160) |
| | LPH2, RPH2 | 1.149 (1.098-1.201) | 10 841 | 1.130 |
| | | 1.117 (1.076-1.159) | 15 490 | (1.099-1.161) |
| 3 | LPH3, H3 | 1.150 (1.106-1.195) | 14 681 | 1.141 |
| | | 1.128 (1.077-1.182) | 10 068 | (1.109-1.174) |
| | LPH3, RPH3 | 1.150 (1.107-1.194) | 14 980 | 1.133 |
| | | 1.115 (1.072-1.159) | 13 961 | (1.103-1.163) |

[a] Potency of RPH relative to H:
Assay 1 1.008 (0.980-1.036) Assay 2 0.999 (0.973-1.025) Assay 3 0.993 (0.966-1.021)

[b] Validity: No significant departure from parallelism and no significant curvature.

Test for Heterogeneity:

| | LPH; H | | | LPH; RPH | | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | Degrees of freedom | Significance [c] | $\chi^2$ | Degrees of freedom | Significance [c] |
| Within ampoules | 1.65 | 3 | NS | 2.21 | 3 | NS |
| Between ampoules | 2.31 | 2 | NS | 5.64 | 2 | NS |
| Total | 3.96 | 5 | NS | 7.85 | 5 | NS |

[c] NS = not significant.

*Annex 2 (continued)*

LABORATORY 4

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) [a] | Weight |
|---|---|---|---|
| 1 | H1, RPH1 | 0.997 (0.942-1.054) | 7 263 |
| 2 | H1, RPH2 | 0.996 (0.946-1.048) | 8 837 |
| 3 | H2, RPH3 | 0.994 (0.958-1.031) | 17 192 |
| 4 | H2, RPH4 | 0.998 (0.946-1.054) | 7 794 |
| 5 | H3, RPH5 | 0.993 (0.942-1.048) | 8 081 |
| 6 | H3, RPH6 | 0.995 (0.943-1.049) | 8 126 |

[a] Validity: No significant departure from parallelism and no significant curvature.

Test for Heterogeneity:

| $\chi^2$ | Degrees of freedom | Significance |
|---|---|---|
| 0.0259 | 5 | Not significant |

LABORATORY 5

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) | Weight | Validity [a] | Combined potency (and 95 % confidence interval) |
|---|---|---|---|---|---|
| 1 | H1, RPH1 | 0.973 (0.941-1.007)<br>1.023 (0.982-1.066)<br>0.976 (0.943-1.009) | 18 896<br>12 946<br>19 019 | | 0.987<br>(0.967-1.007) |
| 2 | H2, RPH2 | 1.045 (1.010-1.080)<br>1.046 (1.013-1.079) | 18 804<br>21 418 | C : P<0.05 | 1.045<br>(1.022-1.069) |
| 3 | H3, RPH3 | 0.975 (0.938-1.014)<br>1.033 (0.999-1.068)<br>1.027 (0.988-1.068) | 14 147<br>19 541<br>14 080 | C : P<0.05 | 1.014<br>(0.993-1.034) |

[a] C = combined curvature significant (P<0.05).
No significant departure from parallelism.

Test for Heterogeneity:

| | $\chi^2$ | Degrees of freedom | Significance |
|---|---|---|---|
| Within ampoules | 12.59 | 5 | P<0.05 |
| Between ampoules | 13.53 | 2 | P<0.01 |
| Total | 26.69 | 7 | P<0.001 |

*Annex 2 (continued)*

LABORATORY 6

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) [a] | Weight | Combined potency (and 95 % confidence interval) |
|---|---|---|---|---|
| 1 | H1, RPH1 | 1.020 (0.977-1.065)<br>0.992 (0.937-1.050) | 12 376<br>7 143 | 1.010<br>(0.977-1.044) |
| 2 | H2, RPH2 | 0.997 (0.842-1.179) [b]<br>1.029 (0.848-1.248) [b] | 824<br>625 | 1.010<br>(0.894-1.142) |
| 3 | H3, RPH3 | 1.075 (0.924-1.251) [b]<br>0.990 (0.824-1.191) [b] | 1 005<br>680 | 1.040<br>(0.928-1.165) |
| 1 | LPH1, RPH1 | 1.144 (1.096-1.193)<br>1.139 (1.094-1.185) | 12 829<br>14 501 | 1.141<br>(1.110-1.174) |
| 2 | LPH2, RPH2 | 1.308 (1.059-1.616)<br>1.157 (0.934-1.433) [b] | 516<br>512 | 1.230<br>(1.064-1.423) |
| 3 | LPH3, RPH3 | 0.986 (0.776-1.253) [b]<br>1.151 (1.075-1.232) [b] | 405<br>4 936 | 1.138<br>(1.067-1.213) |

[a] Validity: No significant departure from parallelism and no significant curvature.
[b] Latin squares incomplete owing to indeterminate zone diameters.

Test for Heterogeneity:

| | H; RPH | | | LPH; RPH | | |
|---|---|---|---|---|---|---|
| | $X^2$ | Degrees of freedom | Significance [c] | $X^2$ | Degrees of freedom | Significance [c] |
| Within ampoules | 0.77 | 3 | NS | 3.02 | 3 | NS |
| Between ampoules | 0.25 | 2 | NS | 1.09 | 2 | NS |
| Total | 1.02 | 5 | NS | 4.11 | 5 | NS |

[c] NS = not significant.

LABORATORY 7

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) [a] | Weight |
|---|---|---|---|
| 1 | H1, RPH1 | 1.017 (0.926-1.117) | 2 610 |
| 2 | H2, RPH2 | 1.064 (0.994-1.139) | 4 959 |
| 3 | H3, RPH3 | 1.099 (1.045-1.156) | 9 587 |

[a] Validity: No significant departure from parallelism.

Test for Heterogeneity:

| $x^2$ | Degrees of freedom | Significance |
|---|---|---|
| 2.54 | 2 | Not significant |

*Annex 2 (continued)*

LABORATORY 8

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) | Weight |
|---|---|---|---|
| 1 | H1, RPH3 | 0.917 (0.882-0.953) | 13 240 |
| 2 | H2, RPH4 | 0.914 (0.816-1.025) | 1 559 |
| 3 | H2, RPH5 | 0.955 (0.856-1.067) | 1 675 |
| 4 | H3, RPH5 | 1.037 (0.966-1.114) | 4 032 |
| 5 | H3, RPH6 | 1.102 (1.054-1.154) | 9 911 |

Test for Heterogeneity:

| $x^2$ | Degrees of freedom | Significance |
|---|---|---|
| 40.27 | 4 | $P<0.001$ |

LABORATORY 9

| Assay | Mean potency ratio | Weight |
|---|---|---|
| 1 | 1.006 | 2 895 (8 tests) |
| 2 | 1.001 | 5 823 (12 tests) |

Test for Heterogeneity:

| $x^2$ | Degrees of freedom | Significance |
|---|---|---|
| 0.019 | 1 | Not significant |

*Annex 2 (continued)*

LABORATORY 10

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) | Weight | Validity[a] |
|---|---|---|---|---|
| 1 | H1, RPH1 | 1.017 (0.978-1.058) | 14 182 | C : P<0.001 |
| 2 | H2, RPH1 | 1.000 (0.960-1.042) | 12 845 | |
| 3 | H3, RPH1 | 0.993 (0.957-1.030) | 16 072 | C : P<0.05 |
| 4 | H1, RPH2 | 1.018 (0.980-1.057) | 15 412 | |
| 5 | H2, RPH2 | 1.014 (0.980-1.050) | 18 120 | C : P<0.05 |
| 6 | H3, RPH2 | 1.014 (0.970-1.060) | 11 142 | |
| 7 | H1, RPH3 | 1.007 (0.962-1.055) | 10 226 | |
| 8 | H2, RPH3 | 1.007 (0.972-1.044) | 17 152 | |
| 9 | H3, RPH3 | 1.011 (0.975-1.047) | 17 435 | |
| 1 | LH1, RPH1 | 1.031 (0.997-1.067) | 18 729 | |
| 2 | LH2, RPH1 | 1.021 (0.981-1.063) | 13 644 | |
| 3 | LH1, RPH2 | 1.039 (1.005-1.075) | 19 358 | |
| 4 | LH2, RPH2 | 1.031 (1.001-1.062) | 25 302 | |
| 5 | LH1, RPH3 | 1.022 (0.990-1.055) | 21 955 | C : P<0.001 |
| 6 | LH2, RPH3 | 1.026 (0.995-1.057) | 23 734 | |

[a] C = combined curvature significant.

Test for Heterogeneity:

| H; RPH | | | LH; RPH | | |
|---|---|---|---|---|---|
| $\chi^2$ | Degrees of freedom | Significance | $\chi^2$ | Degrees of freedom | Significance |
| 1.58 | 8 | Not significant | 0.69 | 5 | Not significant |

LABORATORY 11

| Assay | Ampoules | Potency ratio (and 95 % confidence interval) | Weight | Validity [a] |
|---|---|---|---|---|
| 1 | H1, RPH1 | 1.006 (0.975-1.039) | 22 962 | C : P<0.001 |
| 2 | H2, RPH1 | 1.000 (0.976-1.024) | 39 904 | C : P<0.001 |
| 3 | H3, RPH1 | 1.013 (0.993-1.033) | 59 400 | C : P<0.001 |
| 4 | H1, RPH2 | 1.000 (0.976-1.024) | 39 904 | C : P<0.05 |
| 5 | H2, RPH2 | 1.006 (0.975-1.038) | 23 895 | C : P<0.01 |
| 6 | H3, RPH2 | 1.018 (0.989-1.049) | 26 918 | D : P<0.05 C : P<0.001 |
| 1 | X1, RPH3 | 1.168 (1.141-1.196) | 41 477 | C : P<0.001 S : P<0.001 |
| 2 | X2, RPH3 | 1.045 (1.022-1.068) | 48 473 | D : P<0.001 |

[a] C = combined curvature significant.
S = separate curvatures significant.
D = significant departure from parallelism.

Test for Heterogeneity:

| | $\chi^2$ | Degrees of freedom | Significance |
|---|---|---|---|
| H; RPH | 1.78 | 5 | Not significant |