The Biological Assay of Penicillin by a Modified Plate Method

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SUMMARY: The general form of assay for streptomycin described by Brownlee, Delves, Dorman, Green, Grenfell, Johnson & Smith (1948), based on an 8×8 quasi-Latin square layout of cavities on a large agar-covered glass plate, in place of Petri dishes, can be adapted to penicillin. A new design, using the principle of double confounding, allows the estimation by the usual four-point assay, of seven (instead of three) unknown solutions per plate of 64 holes. In routine use this assay gives fiducial limits ($P=0.95$) of about $\pm 9.6\%$, including dilution errors.

The assay procedure previously described in this Journal (Brownlee *et al.* 1948) for streptomycin, is immediately adaptable to penicillin. For dealing with large numbers of fermenter samples in particular, we have found modifications desirable, which we describe in the present paper.

Experimental design

The basic plate takes 64 holes arranged in an 8×8 square. For the streptomycin assay, Yates's (1937) arrangement of a quasi-Latin square was used to assay three unknown solutions in terms of a standard, all solutions going on the plate at two dilutions in a certain order designed to eliminate bias arising from the time taken to complete the filling of the plate. A slightly less accurate assay was desired which would allow us to test considerably more than three 'unknowns' per plate. The solution of the problem in statistical design is as follows.

Fisher (1942) gives an account of the theory of double confounding, in which two restraints can be imposed on the layout of the factorial experiment. For the 2^6 experiment, Brownlee (1948) gives the most generally useful pair of sets of interactions for double confounding in 8 blocks of 8 and 8 blocks of 8 as

$PQRS, RSTU, PQTU, PRT, QST, PSU,$ and $QRU,$
and $PQRU, PRST, QSTU, PQS, RSU, RQT,$ and $PTU.$

Here the two categories of blocks will correspond to rows and to columns.

If the three factors P, R and S are allocated to denote the 8 levels of a single factor corresponding to 8 solutions (one standard and seven 'unknowns'), T to denote dilution, Q to denote time of filling (Initial I and Final F), and U to be a dummy factor, then a design is arrived at which is satisfactory in its essentials; for the factors used in calculation of potency, namely the 7 degrees of freedom (P, R, S, PR, RS, SP, PRS) corresponding to solutions, and the 1 degree of freedom (T) corresponding to dilution, will all be unconfounded. The defect of this design is that 2 out of the 7 degrees of freedom for differences in slope (the interaction between solutions and levels) are confounded, and

therefore it is difficult to carry out an exact sensitive test for parallelism. However, any gross non-parallelism would show itself, and could be checked by a repeat assay of the suspected solution alone against the standard.

Table 1 shows the layout in accordance with the principle of double confounding, with a typical set of results inserted. The design has been randomized with the restriction imposed that in any one row or column the high and low

Table 1. *Selected layout for the assay of seven unknown solutions of penicillin in a 64-hole plate; with a typical set of results (units in millimetres on the enlarger with magnification $\times 6$)*

6_L^I 110	2_H^F 160	S_L^F 120	1_H^I 168	3_L^I 117	4_H^I 155	5_L^F 110	7_H^F 163
S_H^I 165	4_L^F 113	6_H^F 150	7_L^I 113	5_H^I 155	2_L^I 110	3_H^F 152	1_L^F 120
5_L^I 116	1_H^F 160	3_L^F 110	2_H^I 152	S_L^I 117	7_L^I 155	6_L^F 103	4_H^F 157
3_H^I 160	7_L^F 113	5_H^F 150	4_L^I 108	6_H^I 145	1_L^I 114	S_H^F 160	2_L^F 115
1_L^F 120	5_H^I 155	7_L^I 113	6_H^I 147	4_L^I 110	3_H^F 150	2_L^I 112	S_H^I 165
7_H^F 160	3_L^I 106	1_H^I 159	S_L^F 107	2_H^F 148	5_L^F 100	4_H^I 150	6_L^I 108
2_L^F 117	6_H^I 150	4_L^I 110	5_H^F 143	7_L^F 110	S_H^F 155	1_L^I 113	3_H^I 156
4_H^F 155	S_L^I 120	2_H^I 160	3_L^F 110	1_H^F 160	6_L^I 102	7_H^I 155	5_L^I 110

concentrations alternate; one of the reasons for this procedure is that it reduces the risk of any two inhibition zones overlapping. Of the few arrangements possible with this restriction, that selected has the initial and final fillings alternating in columns in sets of four. We justify this departure from pure randomization on the grounds that (a) the mental strain of filling a large number of plates would otherwise be intolerable, and (b) since every plate is different the ground can, as it were, be considered as being randomized underneath the design.

Details of procedure

To carry out about 70 assays per day 12 plates are required, allowing for a surplus of two in case of accident. For a dozen 150 ml. lots, about 2 l. of assay medium (Evans peptone, 10 g.; Yeastrel, 1.5 g.; Lemco, 3 g.; NaCl, 3.5 g.; Davis agar, 20 g.; distilled water to 1 l., pH 7.0–7.2 before autoclaving) are required.

To obtain it in a perfectly clear state, the medium is first autoclaved in bulk at 20 lb. pressure for 20 min., when some precipitation occurs, filtered through cotton-wool, divided into 150 ml. lots and re-autoclaved at not more than 10–12 lb. pressure for 30 min. to avoid the formation of more precipitate which would affect the clearness of the zones. Sufficient medium for a week's work can be made up at one time. The 12 flasks containing 150 ml. of medium for the day's assay are melted in a steamer, cooled to about 50° and then stood in a water-bath at 45–50° until the temperature is constant, when they are ready for inoculation.

The test organism, *Staphylococcus aureus* 209 P (N.R.R.L./313) is maintained on yeast beef agar slopes (beef extract, 1.5 g.; yeast extract, 2.0 g.; peptone,

6.0 g.; glucose, 1.0 g.; agar, 15 g.; distilled water to 1 l., pH 8.0 before autoclaving at 15 lb. pressure for 20 min.), transferred each evening and incubated overnight at 37°. From this a subculture is made each evening in nutrient broth (peptone, 5.0 g.; yeast extract, 1.5 g.; beef extract, 1.5 g.; NaCl, 3.5 g.; K_2HPO_4 , 3.68 g.; KH_2PO_4 , 1.32 g.; glucose, 1.0 g.; distilled water to 1 l., pH 7.0 before autoclaving at 15 lb. pressure for 20 min.), which is incubated overnight at 37°. The broth is adjusted to an opacity between 1 and 2 on Brown's scale (Burroughs, Wellcome and Co. Ltd. opacity tubes), and 0.3 ml. seeded into 150 ml. of assay medium.

The plates and frames are wiped over with acid ethanol (3 % HCl in 95 % ethanol), which is an adequate means of sterilization, and held at 50° for 1–2 hr., to dry them and to bring them up to the required temperature for pouring. The medium and plates should be at roughly the same temperature to ensure that the medium spreads evenly over the plates before it begins to set. When poured, the plates are placed on a level table, where they may be stacked two or three deep, and left for about an hour. The cavities are then cut with a no. 5 cork borer, previously sterilized by dipping in ethanol and flaming, and the agar plugs picked out with a spear-headed dissecting needle similarly sterilized. A multiple borer may be used which cuts all the cavities at once, but this must be kept well sharpened, otherwise the cavities become irregular.

The penicillin standard is a sodium salt of penicillin *G*, 1667 units/mg.; a 320 units/ml. solution is made up each day in pH 6.0 buffer (50 ml. 0.1 M- KH_2PO_4 + 5.64 ml. 0.1 M-NaOH made up to 100 ml.) and from this the 1.6 units/ml. and 0.4 unit/ml. solutions, used as the S_H and S_L respectively, are made up in pH 7.0 buffer (50 ml. 0.1 M- KH_2PO_4 + 29.54 ml. 0.1 M-NaOH made up to 100 ml.). The previous day's standard is also put on some plates. It has been found to deteriorate by about 4 % in the 24 hr., and is therefore not suitable for use as a standard; but is a safeguard in case anything should go wrong with the current day's standard.

All fermentation samples are first centrifuged or filtered. Three stages of dilution then follow. The first stage is the same for all samples: 5 ml. are diluted in a 50 ml. volumetric flask with pH 6.0 buffer; this gives a 1 in 10 dilution. The second stage depends on the expected titre of the sample; according to whether this is approximately 160, 240, 320, 480 or 640 units/ml., the dilution used is 5 in 50, $3\frac{1}{2}$ in 50, 5 in 100, $3\frac{1}{2}$ in 100 or 5 in 200 respectively in pH 7.0 buffer; this gives the high concentration of the sample, i.e. 1_H , 2_H , etc. The next stage gives the low concentration, 1_L , 2_L , etc., and is again the same for all samples. Two ml. of the *H* solution are added to 6 ml. of pH 7.0 buffer, previously run into a sample bottle by means of an automatic filler. The *H* and *L* concentrations of each sample are now in sample bottles ready for filling. All pipettes, sample bottles and centrifuge tubes are sterilized in an oven before use, and volumetric flasks are autoclaved, with the exception of those used for making up standards, which are rinsed with sulphuric acid.

Each operator has her own filler and this must be used throughout a plate, as the fillers vary slightly in the volume they deliver, from about 0.05 to 0.06 ml., and it is important that a constant volume should be delivered each

time. The filler is rinsed with pH 7.0 buffer between all solutions, including the *H* and *L* of the same sample. The time taken to fill a plate is about 10–15 min. and in order to balance the time effect, which is evident after even half this period, two out of the four cavities for each solution are filled first, starting with the S_H^I, S_L^I followed by sample $1_H^I, 1_L^I$, and finishing with sample $7_H^I, 7_L^I$; the order is then reversed and the second two cavities for each solution are filled, starting with sample $7_L^F, 7_H^F$ and returning to sample $1_L^F, 1_H^F$ and so back to S_L^F, S_H^F .

The plates are incubated overnight at 37°. It is advisable to distribute the plates on different shelves of the incubator, if possible not more than two deep, because packing increases the amount of condensation, favouring contamination which interferes with the clearness of the zones. The zones are projected for measurement on a screen, magnified six times (Brownlee *et al.* 1948). When finished with, the plates and frames are soaked in disinfectant for at least an hour before washing.

Calculation of the potency ratio

The usual formula for the potency ratio is $\text{antilog } dD/B$, where d is the logarithm of the dilution ratio, and

$$D = U_H + U_L - S_H - S_L$$

and

$$B = U_H - U_L + S_H - S_L,$$

where U_H, S_H denote the sums of the readings (here four) for the higher concentration of the unknown and standard respectively and U_L, S_L the sums for the two lower concentrations. Both $U_H - U_L$ and $S_H - S_L$ are estimates of the slope of the response curve for this particular plate, but in order to get the best estimate we can make use of all seven values of $U_H - U_L$ along with $S_H - S_L$. We should therefore use for B'

$$\frac{1}{4} \left[(S_H - S_L) + \sum_1^7 (U_H - U_L) \right].$$

The accuracy of the assay in practice

The analysis of variance of the typical assay shown in Table 1 is given in Table 2. It will be noted from the row and column mean squares, which are large in comparison with the residual mean square, how extremely effective the double confounding arrangement is in removing plate heterogeneity.

Table 2. *Analysis of variance of results in Table 1*

Source of variance	Degrees of freedom	Sum of squares	Mean square
Rows	7	307.25	43.89
Columns	7	415.50	59.36
Levels	1	30,537.56	30,537.56
Solutions	7	977.50	139.64
Solutions $\times$ Levels	5	19.31	3.86
Time	1	42.25	42.25
Solutions $\times$ Time	5	21.75	4.35
Residual	30	155.88	5.196
Total	63	32,477.00	

In this particular plate the residual variance s^2 is 5.196. To calculate the internal error of an assay in which the potency of the unknown is close to that of the standard, we use the approximate formula for the standard error s_m of the logarithm of the potency ratio, which is

$$s_m = \frac{2ds \sqrt{n}}{B'},$$

where d is the logarithm of the dilution ratio, n is the number of observations at each of the four points of the assay. Here $n=4$, $d=0.6021$ and $B'=349.5$ and hence $s_m=0.01548$, with 30 degrees of freedom.

The 95 % fiducial limits for the logarithm of the potency ratio are ± 0.03204 and thus the limits for the potency ratio are 92.9 and 107.7 %. These limits, however, are based on only the internal error of a plate and we are more interested in external estimates of error, which contain dilution errors, etc. The following experiment was designed to obtain such estimates.

Table 3. *Sets of penicillin assays carried out to determine magnitude of experimental errors*

Dilution of sample	Operator and Plate no.							
	A		B		C		D	
	1	2	3	4	5	6	7	8
	units/ml.							
1/150	323	294	300	299	300	282	319	317
	370	329	312	279	306	285	290	288
1/200	299	303	316	321	302	287	317	314
	317	297	319	303	296	290	314	310
1/300	302	309	310	307	328	284	312	320
	306	317	302	302	323	293	301	301

The results of a series of assays to measure the overall error are given in Table 3. Four workers each made up dilutions in duplicate of a standard solution at dilutions of 1/150, 1/200, and 1/300. This gave six solutions for each worker and these were each assayed on two plates. Thus one of the two dilutions at 1/150 made up by operator A gave 323 on the first of her plates and 294 on her second. Her second dilution at 1/150 gave 370 on her first plate and 329 on her second. The seventh position was left blank. The analysis of variance of the results is in Table 4. The variance of a single assay is given by the sum of the components of variance in the last column, viz. 273.47, and the estimated 95 % limits of error for a single assay (including dilution errors) are ± 10.8 %. In this set of results the errors in repeat dilutions are rather large; they may have arisen from inadequate shaking of the flasks.

This standard of accuracy can be attained in practice. Over several days 61 fermenter vessels were sampled and assayed in duplicate. The range between duplicates corresponded to 95 % error limits of ± 9.6 %. Should greater accuracy than this be required, it can be readily obtained by replication, the error limits of course being inversely proportional to the square root of the

number of replications. It is obvious that the replication should take place on different plates. Sevenfold replication, equivalent on the average to one unknown per plate, would give 95 % fiducial limits of 3.65 %.

Table 4. *Analysis of variance of results in Table 3*

Source of variance	Degrees of freedom	Sum of squares	Mean square	Component of variance
Operators	3	1,569.56	523.187	—
Dilutions	2	18.00	9.000	—
Operators $\times$ Dilutions	6	2,696.50	449.417	—
Plates within Operators	4	2,074.42	518.605	55.327*
Plates within Operators $\times$ Dilutions	8	1,508.83	188.604	67.625
Between repeat Dilutions within Operators	12	2,972.25	247.687	97.167
Residual	12	640.25	53.354	53.354
Total	47	11,479.81	—	—

* In calculating this component of variance the Operators Mean Square has been pooled with that for Plates within Operators.

To compare the relative efficiency of these large plates with that of Petri dishes is not easy. Considering merely the number of holes to be cut, filled and measured, this sevenfold replication is equivalent to sixteen Petri dishes per unknown, but with Petri dishes we have never been able to obtain 95 % fiducial limits anything like as low as 3.65 %. A single plate is also probably more easily handled, poured, washed, etc., than sixteen Petri dishes. It has been our experience that four operators can deal with 10 plates per day, i.e. testing 70 unknown solutions.

It will be noted that the error between assays on the same plate is less than the error between assays on different plates, and therefore when one is engaged in experiments comparing different treatments, and not immediately concerned with the absolute value, it is advantageous to arrange the treatments so that they occur in blocks of seven, if necessary using the balanced incomplete block designs, a table of which is given by Fisher & Yates (1943).

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