RESEARCH PAPERS

A METHOD FOR THE DETERMINATION OF pA_2 AT TWO MINUTES

By Mary F. Lockett and A. L. Bartlet

From the Department of Physiology and Pharmacology, Chelsea Polytechnic, Manresa Road, London, S.W.3

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The systematic quantitative study of the actions of drugs on specific drug receptor surfaces in isolated tissues was initiated in this country by A. J. Clark. Work by Clark and his collaborators[1–4] and by Gaddum[5,6] established the existence of a number of different types of cell-surface receptors. Each type was specifically fitted by a naturally occurring key or activating drug, of high potency, capable of producing characteristic action on the tissue. The perfect fit of the key drug for the lock or receptor was believed to result from the chemical configuration of both, and it was therefore expected that the affinity between drug and receptor would be higher for the key than for any other drug. Key drugs appeared to occupy their receptors reversibly and according to the law of mass action, and the tissue responses evoked were considered proportional to the percentage of specific receptors occupied. In accordance with theory, drugs were found amongst compounds chemically related to key drugs, which themselves produced no action on the isolated tissues, but which made these tissues less sensitive to the action of the related key drug. These inhibitor compounds were apparently attracted to the receptors and occupied them, less adequately than the key drug perhaps since no tissue activation occurred, and usually less reversibly than the key drug. When such an inhibitor and the key drug were allowed to act on the tissue together, both competed for the receptors, and did so according to the law of mass action; therefore, a higher concentration of key drug was required to produce a given effect in the presence of the inhibitor than in its absence. Such specific competitive antagonists of key drugs have already proved their value clinically in combating, for instance, the unwanted effects of naturally occurring key drugs, such as histamine, acetylcholine, and adrenaline.

Schild[7] introduced pA_x as a measure of the strength with which such an inhibitor drug competes with a key drug in any one isolated system. He defined pA_x as "the negative logarithm to the base 10 of the molar concentration of an antagonistic drug which will reduce the effect of a multiple dose, $_x$, of active drug to that of a single dose." Since the effect of these inhibitor drugs varies with time, the value pA_x is determined after a stated interval of contact between tissue and inhibitor drug, usually 2 minutes.

The reliability of this pA scale for assessment of the relative potency of synthetic inhibitor drugs is so widely accepted that need has arisen for a

simple quick method of pA_2 ($x = 2$) determination at 2 minutes, which allows the calculation of limits of error from the internal data of the experiment. The object of this paper is to present such a method. A detailed description of the method is given first, and this is followed by evidence which must be provided in support of its validity.

The Method

A strip of guinea-pig ileum is suspended in oxygenated Tyrode's solution at a temperature between 32° and 34°C., in a bath of known and constant volume. A suitable dose, k, of key drug is found by trial; this dose should produce an effect which is approximately 50 per cent. of the maximum. Dose k is repeated every 3 minutes, and is allowed to act for a fixed contact period, e.g., 30 seconds before the bath fluid is changed. When the response to k has been constant, or nearly so, for three consecutive contractions, a dose of inhibitor is added to the bath exactly 2 minutes before the next dose of key drug is due. At the end of this 2 minutes, double the usual dose, $2k$, of key drug is added without washing out; it is allowed to exert its effect in the presence of the inhibitor throughout the usual contact period for key drug; then the two are washed out together. Return is then made to the original dose, k, of key drug; it is added to the bath every 3 minutes for the same fixed contact period as before, until the tissue recovers from the effect of the inhibitor, and settles down again to a constant response. Then, after 3 control contractions in response to dose k, the inhibitor drug may be tested again.

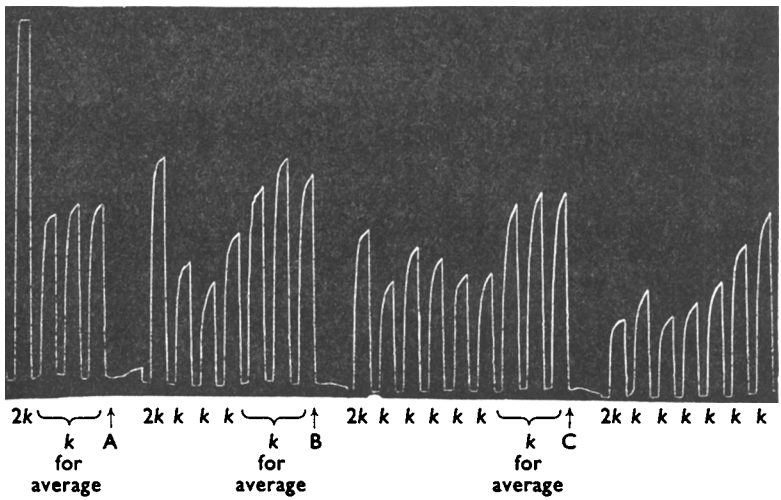

FIG. 1. Determination of pA_2 at 2 min. The tracing was made by guinea-pig ileum contracting in a 20 ml. bath of Tyrode's fluid at 32° C. See text for explanation.

After a few preliminary trials, three doses, A, B, and C of inhibitor are selected. These doses A, B, and C are chosen as weights of inhibitor which increase in series as simple powers of 2 or 3. The effect of dose $2k$ of key drug after A should be greater than that of k, and less than that of

$2k$ in the absence of inhibitor. The effect of dose $2k$ after C should be less than that of k in the absence of inhibitor, but must be readily measurable. B will produce an inhibition of $2k$ intermediate between those due to A and C.

Doses A, B, and C of inhibitor are then given, exactly as described above, in the order of a 3 × 3 Latin Square (e.g., ABC, BCA, CAB).

The procedure is illustrated in Figure 1. The tracing is part of a record obtained by a technician who was asked to estimate pA_2 at 2 minutes for histamine-diphenhydramine, and who was given only the above written instructions. The doses selected were:—k, 1 μg. histamine; A, B, and C, 0·07, 0·14, and 0·28 μg. of diphenhydramine respectively (these increase as simple powers of 2). The bath was calibrated to a volume of 20 ml. The drum was stopped each time when the key drug was washed out, and was restarted either 30 seconds before the addition of key drug, or, when inhibitor was used, 30 seconds after its addition to the bath. The figure shows the first line of a completed Latin Square. The first response recorded is to $2k$, the second to k, in the absence of any inhibitor. Three control responses to k were followed by the addition of A to the bath. A small spontaneous contraction occurred during the 2 minutes contact of the tissue with diphenhydramine before the addition of $2k$ to the bath and was ignored. Whereas the three control responses to k were satisfactory before the addition of inhibitor doses A and C, they were not so before the addition of B; the tissue had probably not fully recovered from the action of A by the first of the three control doses of k, and had not settled down to give a constant, or nearly constant, response to k.

When the whole Latin Square had been completed the heights of the relevant contractions were measured. On each occasion when diphenhydramine had been used, the difference between the height of the response to $2k$ and the average height of the three preceding control responses to k was expressed as a plus or minus percentage of that average. The results obtained are shown in Table I.

TABLE I

SUMMARY OF RESULTS FROM A SINGLE DETERMINATION OF pA_2 AT 2 MINUTES FOR DIPHENHYDRAMINE-HISTAMINE ON GUINEA-PIG ILEUM

Dose of diphenhydramine	μg.	Effect of diphenhydramine $\frac{(100(2k-k))}{k}$ Individual trials			Average
A	0·07	+30	+23	+16	+23
B	0·14	−23	−34	−33	−30
C	0·28	−60	−74	−70	−68

k = average control response to 1 μg. histamine; $2k$ = response to 2 μg. histamine in the presence of diphenhydramine.

The pA_2 value for histamine-diphenhydramine at 2 minutes was then determined both graphically, and by calculation.

pA_2 determined graphically. The average percentage differences between the responses to $2k$ after and k before diphenhydramine (Table

I) were plotted as ordinates against the logarithms of the doses of antihistamine as abscissæ. The line of best fit for the resulting 3 points was drawn by eye (Fig. 2); then the log-dose corresponding to zero percentage difference was read from the graph (2·91). Its antilogarithm (0·096) gave the μg. of antihistamine which, when added to the bath volume (V = 20 ml.), would have produced a molar concentration the negative logarithm of which was pA_2 diphenhydramine-histamine at 2 minutes.

The molarity resulting from the addition w μg. of drug to a bath volume of V ml. is given by the following equation:—

$$\text{Molarity} = \frac{w}{\text{M.W.} \times \text{V} \times 1000}, \text{ where M.W. stands for molecular weight.}$$

Hence the molarity resulting from the addition of 0·096 μg. of diphenhydramine (M.W. 255) to a bath volume of 20 ml. was $1{\cdot}87 \times 10^{-8}$. The pA_2 value required was given by the negative logarithm of this figure. A negative logarithm of any number is the logarithm of the reciprocal and so can be found by subtracting the logarithm of the original number from the logarithm of 1·0. The value for pA_2 diphenhydramine-histamine at 2 minutes given by this experiment was, therefore, $0{\cdot}0000 - \bar{8}{\cdot}2724 = 7{\cdot}7$.

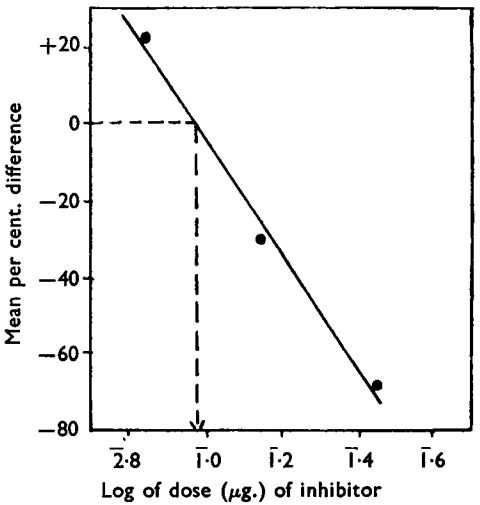

FIG. 2. Graphical determination of the log-dose of inhibitor which, when added to the bath volume, will, in two minutes, reduce the effect of a double dose of activating drug to that of a single dose. Ordinates: average percentage difference between responses to a double dose of activating drug in the presence of inhibitor, and to a single dose of activating drug in the absence of inhibitor.

pA_2 by calculation. The first object of this procedure was to find the line of best fit for the 3 points in Figure 2 exactly, instead of approximately by eye. The method used was the standard one for the calculation of regression lines where the response is graded. Its application is briefly explained.

The equation for any straight line is: $y = a + bx$. (1) a and b are constants; b gives the slope of the line. y refers to the response and is dependent on x, the log-dose.

Let Σ stand for "the sum of", and $\bar{x}$ and $\bar{y}$ for the means of all values for x and y respectively.

Since the line of best fit for the 3 points will pass through the means $\bar{x}$ and $\bar{y}$ with a slope b, and will be such that $(\bar{y}-y) = O$, and $(\bar{y}-y)^2$ is a minimum, $b = \dfrac{(\bar{x}-x)(\bar{y}-y)}{(\bar{x}-x)^2}$. The calculation of $\bar{x}$, $\bar{y}$, and b was most

easily made in tabular form (Table II). In order to reduce the arithmetic the actual doses of inhibitor used are multiplied by some suitable fraction to convert them to 2, 4, and 8 or 3, 9 and 27. In this case the doses of diphenhydramine in μg. (column 1) were first multiplied by 100/3·5 (column 2), and were then converted to logarithms to the base 2, giving

TABLE II

CALCULATION OF THE LINE OF BEST FIT FROM THE DATA IN TABLE I

Dose of diphenhydramine				Calculation of slope (b)			
μg.	μg. × 100/3·5	μg. × 100/3·5 as log. to base 2·0	Response per cent. change				
		x	y	$\bar{x}-x$	$\bar{y}-y$	$(\bar{x}-x)(\bar{y}-y)$	$(\bar{x}-x)^2$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8) column
0·07	2·0	1	+23	+1	−48	−48	+1
0·14	4·0	2	−30	0	+5	0	0
0·28	8·0	3	−68	−1	+43	−43	+1
		$\Sigma x = 6$	$\Sigma y = -75$			$\Sigma = -91$	$\Sigma = +2$
		$\bar{x} = 2$	$\bar{y} = -25$				

$$b = \frac{(\bar{x}-x)(\bar{y}-y)}{(\bar{x}-x)^2} = \frac{91}{2} = -45{\cdot}5$$

$\bar{y} = a + b\bar{x}$, therefore $a = 66{\cdot}0$.

values for x (column 3). The corresponding values for y, which were the averages of the percentage differences between the responses to $2k$ and k at each dose level of diphenhydramine (Table I) are listed in column 4. $\bar{x}$ and $\bar{y}$ are calculated in columns 3 and 4 respectively and b in columns 5 to 8. b is given by the sum of column 7 divided by that of column 8.

These calculated values for $\bar{x}$, $\bar{y}$, and b were then used to find the value for a, by substitution into equation (1).

Since a and b were now known, the value of x corresponding to any given value of y could be found from equation (1). The value of x required for the estimation of pA_2 was that when $y = O$; i.e., $x = 1{\cdot}451$, which is equivalent to $2^{1{\cdot}451} \times \frac{3{\cdot}5}{100}$ μg. To evaluate $2^{1{\cdot}451}$, let z be the logarithm of the required number to the base 10, then $z = \log_{\cdot 10} 2{\cdot}0 \times 1{\cdot}451 = 0{\cdot}3010 \times 1{\cdot}451 = 0{\cdot}4368$, antilogarithm 2·734. Then the required value of x in μg. was $2{\cdot}734 \times \frac{3{\cdot}5}{100} = 0{\cdot}9560$. The molarity which resulted from the addition of 0·9560 μg. of diphenhydramine to the bath, and the pA_2 value for diphenhydramine-histamine at 2 minutes were then estimated exactly as described in the graphical method. The pA_2 value at 2 minutes obtained by calculation was 7·73.

The error of this estimate was most easily calculated in tabular form (Table III). The values of x (log-dose) and the corresponding observed values of y (average percentage responses) were entered in columns 1 and 2 respectively. The values of y given by the line of best fit were then calculated by substitution into equation (1), and were entered in column

3. Column 4 gave the difference between the observed and calculated values for y and column 5 these differences squared and multiplied by the number (n) of observations averaged to give the observed value of y. The variance (s^2) was obtained by dividing the total sum of squares in column 5 by N-5, where N stands for the total number of trials (5). This reduction of 5 was made because repeated trial had shown the variances so computed barely larger than those obtained by full analysis. It follows from equation (1) that there must be two components of the standard deviation of any value for y, because both a and b may be in error; moreover, any error in b will become magnified as the value for x departs from $\bar{x}$. Since the variance of $b = \frac{s^2}{\Sigma n(\bar{x}-x)^2}$ and the standard error of the mean $= \sqrt{s^2/N}$, the standard deviation (s_y) of any value of y was given by the equation $s_y = s\sqrt{\frac{1}{N} + \frac{(\bar{x}-x)^2}{\Sigma n(\bar{x}-x)^2}}$. It remained only to determine the standard deviation of y at the dose level x corresponding to pA_2 at 2 minutes. When $x = 1{\cdot}451$, $s_y = 4{\cdot}26$.

TABLE III

CALCULATION OF THE ERROR OF THE ESTIMATE OF pA_2 AT 2 MIN.

x	y obs. y_O	y calc. y_C	Difference $y_O - y_C$	Difference$^2 \times n$ $n(y_O-y_C)^2$
(1)	(2)	(3)	(4)	(5)
1	+23	+20·5	+2·5	18·75
2	−30	−25·0	−5·0	75·00
3	−68	−70·5	+2·5	18·75
				$\Sigma = 112{\cdot}5$
				$s^2 = 28{\cdot}125$

Applying fiducial limits this value for s_y was multiplied by the value of t with 4 degrees of freedom at a probability level of 0·95 ($t = 2{\cdot}776$ and $s_y.t = 11{\cdot}83$). The fiducial limits of y when $x = 1{\cdot}451$ were, therefore, $\pm 11{\cdot}83$. Values of x corresponding to these limiting values of y were then calculated by substitution into equation (1); i.e., $x = 1{\cdot}7105$ or $1{\cdot}1905$. The corresponding pA_2 values were calculated as above, and were found to be 7·65 and 7·81, differing from the mean pA_2 value of 7·73 by 0·08 or 1·03 per cent. The fiducial limits (P = 0·95) were therefore $\pm$ 1·03 per cent.

EXAMINATION OF THE METHOD

The method described is valid only because the percentage reduction in the response to $2k$ of key drug in the presence of inhibitor is linearly related to the log-dose of inhibitor for a range exceeding 90 per cent. of the uninhibited response to $2k$. Evidence of this fact was obtained in the course of 21 estimations of pA_2 at 2 minutes for antihistamine in extracts. These estimates were made by the method advocated above except that uninhibited responses to $2k$ were recorded amongst the control responses to k. The percentage differences between the responses to $2k$ before and after antihistamine were plotted as abscissæ, and the differences between the observed and calculated values for y as ordinates. The scatter diagram (Fig. 3) which resulted showed first, that the deviation of any point from linearity never exceeded 10 per cent. of y; secondly, that 86 per cent.

of all points deviated from linearity by less than 5 per cent. of y; thirdly, that departure from linearity was not increased, but tended to decrease, at the limits of the working range (5 per cent. and 95 per cent. inhibition of the response to $2k$). Part explanation for this tendency may be deduced from Figure 4. Here, fiducial limits (P = 0·95), expressed as ± percentages of the mean, have been plotted as ordinates against the percentage range of the $2k$ response employed in each experiment. When widely different degrees of inhibition were produced by the three selected doses of inhibitor, the experiment covered a large percentage range of the uninhibited response to $2k$. Figure 4 indicates that estimates from wide range experiments in this series tend to be more reliable than those from narrow range experiments.

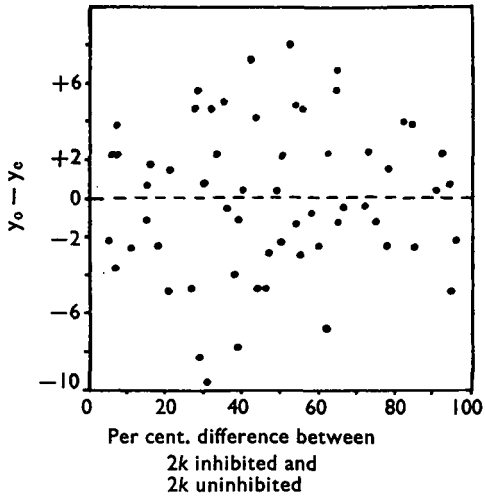

FIG. 3. Investigation of the incidence of deviation from linearity in the regression of y on x during determinations of pA_2 at 2 min. See text for explanation. Ordinates: differences between observed (y) and calculated (y_e) values of y. Abscissæ: percentage differences between responses to 2 k of activating drug, before and after antihistamine.

The fact that the fiducial limits (P = 0·95) encountered in this series of experiments with crude extracts were wider than those recorded when pure drugs were used (cf. Figure 4 and Table IV) is attributed to the presence of other substances with weak pharmacological actions in these extracts together with the antihistamine.

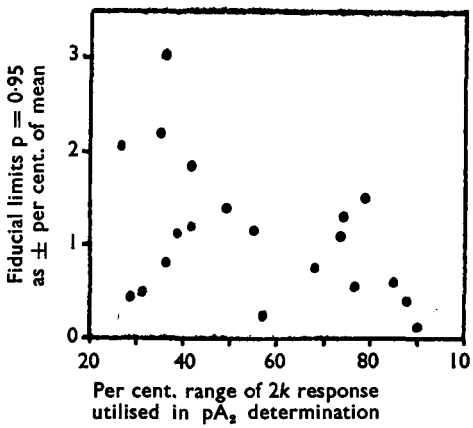

FIG. 4. Calculated fiducial limits related to the range of the response to $2k$ used during pA_2 determinations. Ordinates: fiducial limits (P = 0·095) expressed as ± per cent. mean. Abscissæ: per cent. range of the response to $2k$ utilised during the determination. See text for explanation.

EXAMINATION OF THE RESULTS OBTAINED BY THE METHOD ADVOCATED

Comparison has been made in Table IV of values of pA_2 at 2 minutes obtained for mepyramine-histamine, diphenhydramine-histamine, and atropine-acetylcholine by

Schild[7] in column 1 and by the new method in column 2. In each case the mean value has been entered ± the standard error of the mean followed by the number of experiments within brackets. There is good agreement between the two sets of results. Six values for pA_2 at 2 minutes for atropine-acetylcholine on rat ileum are included in column 2, since these values have not, in our experiments, differed from those obtained using guinea-pig ileum. All other values in this table were obtained with guinea-pig ileum.

TABLE IV

SUMMARY OF pA_2 VALUES OBTAINED, AND OF CALCULATED LIMITS OF ERROR

pA_2 values ± S.E. (N)		Fiducial limits of error (P = 0·95) for method described	
Schild[7] (1)	Method described (2)	In individual experiments (3)	Directly estimated (4)
Mepyramine-histamine 8·71 ± 0·021 (4)	8·711 ± 0·018 (8)	± 0·22 to 2·10 per cent.	± 0·84 per cent.
Diphenhydramine-histamine 7·75 ± 0·035 (8)	7·735 ± 0·090 (6)	± 0·21 to 2·11 per cent.	± 1·46 per cent.
Atropine-acetylcholine 8·27 ± 0·021 (11)	8·246 ± 0·026 (11)	± 0·41 to 2·80 per cent.	± 2·51 per cent.

The error of each estimate of pA_2 at 2 minutes was calculated from the internal evidence of the test; fiducial limits (P = 0·95) were applied and were expressed as ± percentages of the means. The ranges of these limiting values were entered in Table IV, column 3. Direct estimates of the error of the method were made by expressing the difference of each calculated value of pA_2 from its mean as a percentage of that mean, squaring and summing the percentage differences, then dividing by N-1 to obtain the variance in the usual way. The square roots of the variances multiplied by the value of t(P = 0·95) for N-1 degrees of freedom gave the direct estimates of the fiducial limits entered in Table IV, column 5. For each pair of drugs the fiducial limits obtained by direct estimation fell within the range of values calculated from the internal evidence of individual experiments. The small numbers of observations used for the direct estimates of error are undoubtedly largely responsible for this finding. It may, however, be concluded that the fiducial limits obtained for individual estimates of pA_2 are satisfactorily wide.

SUMMARY

1. A method for the determination of pA_2 at 2 minutes has been presented.

2. The method allows the calculation of limits of error from the internal data of the experiment.

3. Fiducial limits of error (P = 0·95) obtained for mepyramine-histamine, diphenhydramine-histamine, and atropine-acetylcholine were always less than ± 3·0 per cent. of the mean.

4. Two such estimates can regularly be made by a skilled worker in seven hours, using only a single, manually operated, isolated organ bath.

This work was carried out whilst A. L. Bartlet was in receipt of a D.S.I.R. award.

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