How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated?

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The potencies of pharmacological competitive antagonists are commonly expressed in terms of pA_2 values. Such estimates are useful in assessing the selectivity of antagonists and also in characterizing pharmacological receptors in different isolated tisues.

The most commonly used method for estimating pA_2 values for pharmacological competitive antagonists is to plot log (DR − 1) against log [I] where DR represents the agonist dose-ratio, [I] represents the molar concentration of the antagonist and the logarithms are to the base 10. This method, introduced by Arunlakshana & Schild (1959), depends on the validity of the theoretical equation

$$\log (DR - 1) = pA_2 + \log [I] \quad .. \quad .. \quad (1a)$$

or $$pA_2 = \log (DR - 1) - \log [I] \quad .. \quad .. \quad (1b)$$

These equations were, in turn, derived from the null equation for competitive antagonism, namely

$$(DR - 1) = K_I[I] \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where K_I is the affinity constant of the antagonist for the receptor.

Theoretically the pA_2 is therefore equal to log K_I, and a plot of log (DR − 1) *vs* log [I] should give a straight line

$$\log (DR - 1) = a_1 + b_1 \log [I] \quad .. \quad .. \quad (3)$$

or $Y = a_1 + b_1X$, where the value of b_1 should not differ significantly from unity *if the antagonism is competitive.*

In the following discussion which is concerned with various methods for estimating affinity constants and pA_2 values, it is assumed that each dose-ratio estimate has been made as accurately as possible from the displacement of essentially parallel log dose/response curves and that adequate practical precautions have been taken to eliminate complications which might arise from uptake or metabolism of the agonist and antagonist, or from non-equilibrium conditions (see e.g. Furchgott, 1972).

The value of a_1 in equation 3 is one estimate of the pA_2, with confidence limits

$$a_1 \pm ts(1/N + (\bar{X})^2/\Sigma(X - \bar{X})^2)^{1/2}$$

where t is Student's t-factor, s is the square root of the mean sum of squares about regression, N is the number of points on the line and $\bar{X}$ is the mean value of log [I]. Since most useful antagonists act at low concentrations and may have 'non-selective' actions at high concentrations the value of $\bar{X}$ will usually lie far from zero. The confidence limits on a_1 are therefore likely to be wide. For this reason the use of the ordinary regression constant, a_1, as an estimate of pA_2 should be avoided.

Provided that b_1 is exactly unity, the pA_2 is also equal to the value of −log[I] when log (DR − 1) is zero, corresponding to a dose-ratio of 2 (see eqn 1a). This latter estimate therefore accords with the experimental definition of pA_2 (Schild, 1947) and for the reasons outlined above usually involves a shorter extrapolation and narrower confidence limits than are involved in the estimation of a_1. If however b_1 is not exactly unity, though not statistically significantly different from unity, then the value of pA_2 estimated from the value of log [I] when log (DR − 1) is equal to zero is not a_1 but a_1/b_1. The approximate confidence limits on this estimate of pA_2 are $a_1/b_1 \pm (ts/b_1)$ $(1/N + (-(a_1/b_1) - \bar{X})^2/\Sigma(X - \bar{X})^2)^{1/2}$.

Therefore a plot of log (DR − 1) *vs* log [I] generally gives two different estimates of pA_2 or of log K_I, namely a_1 and a_1/b_1. What is even more disconcerting is the fact that when error estimates are made on a_1 and on a_1/b_1 the value of a_1 may lie outside the approximate confidence limits of a_1/b_1 and vice versa, even when b_1 does not differ significantly from unity.

For the reasons already discussed the choice between a_1 and a_1/b_1 depends on the position of the experimental points on the plot of log (DR − 1) *vs* log [I]. Of the two a_1/b_1 is generally to be preferred, but neither value gives the best estimate of log K_I for a competitive antagonist. Alternative methods are therefore considered below:

(a) Suppose that the plot of log (DR − 1) *vs* log [I] gives a statistically acceptable straight line with a slope which is not significantly different from unity. It may then be concluded that there is no strong evidence against the assumption that the antagonism is competitive and that deviation from equation 1a is due to chance. It is then reasonable to fit to the data *the best straight line with a slope of unity*, i.e. $Y = a_2 + X$, corresponding to equation 1a. The best estimate of the pA_2, assuming competitive antagonism, is then

$$pA_2 = a_2 = (\Sigma y - \Sigma X)/N$$

where y and X are the experimental values of log (DR −1) and log [I] respectively and N is the number of points. The variance of the pA_2 is then s^2/N where s^2 is the mean sum of squared deviations about the line. The confidence limits of the pA_2 are $a_2 \pm ts/\sqrt{N}$ where t has N-1 degrees of freedom.

(b) Another method, which was also used by Arunlakshana and Schild and which is closely related to that described above, is to convert each experimental value of DR into a pA_2 value using equation 1b. If the antagonism is competitive then the pA_2 values should

be essentially constant regardless of the value of [I] though subject to random error. Therefore such individual pA_2 values may be plotted *vs* log [I] to decide whether there is any significant regression of pA_2 on log [I]. Any regression coefficient significantly different from zero would invalidate the assumption of competitive antagonism and preclude the use of the pA_2 value to characterise the receptor. If however there is no significant dependence of pA_2 on log [I] then the best estimate of pA_2 is the mean value, with appropriate confidence limits.

(c) A third method is to plot (DR − 1) *vs* [I] (see eqn 2). Such a plot should give a straight line $Y = a_3 + b_3X$, where Y and X are now (DR − 1) and [I] respectively. It will be seen from equation 2 that the value of a_3 should not be significantly different from zero if the antagonism is competitive. If this is so then by analogy with the argument used in section (a) the data may be fitted by the straight line $Y = b_4 X$ where b_4 is the best estimate of K_I (see eqn 2). The best values of K_I and of the variance of K_I are then $\Sigma Xy/\Sigma X^2$ and $s^2/\Sigma X^2$ where y is the observed value of (DR − 1) and s^2 is the mean sum of squares about the best line passing through the origin. The confidence limits of K_I are $b_4 \pm ts/(\Sigma X^2)^{1/2}$ with N-1 degrees of freedom.

(d) In method (b) each experimental estimate of DR at any chosen value of [I] provided a single estimate of pA_2 from equation 1b. Similarly each such estimate of DR provides a single estimate of K_I from equation 2. Therefore the arguments put forward in section (b) concerning the estimation of pA_2 values may also be applied, at least approximately, to estimates of K_I.

If pA_2 is regarded as a purely experimental quantity then estimates of a_1/b_1 and of the confidence limits of a_1/b_1 can be made from the regression of log (DR − 1) on log [I]. If however the antagonist is judged to be competitive and an estimate of log K_I or of K_I is required, with confidence limits, then methods (a), (b), or (c) (or to a lesser extent method (d)) seem preferable. In these circumstances if log (DR − 1) has a more constant variance or spread than has (DR − 1), when the concentration of the antagonist is varied, then it is better to use method (a) or (b) rather than (c). The converse also applies.

Examination of reports in the literature of estimates of affinity constants and of pA_2 values shows that in many cases insufficient information is given to decide exactly how such estimates and in particular how error estimates have been made. Other problems arise when comparisons are being made between pA_2 values obtained by different workers or using somewhat different techniques. The possibility of some experimental bias must be kept in mind when such comparisons are made. Although pA_2 estimates have been shown to be reproducible quantities, results of antagonist studies at low dose-ratios may be biased due to drift of tissue sensitivity with time whereas results obtained at high dose-ratios may be in error due to non-specific drug effects. In such circumstances results obtained by one worker using one method may be highly reproducible but nevertheless different from another set of equally reproducible results obtained by a second worker using a slightly different method (see e.g. Abramson, Barlow & others, 1969). Because of the existence of small but apparently significant differences in estimates of the pA_2 values for a given drug, an arbitrary judgement is sometimes made as to the magnitude of the difference in pA_2 which is to be regarded as 'real'. This approach is too subjective to be entirely satisfactory though it has the merit of practical simplicity. It is probably better, whenever possible, to try to eliminate experimental bias by restricting comparisons to results obtained using the same experimental and analytical techniques. This is especially so when differences between results lie near the borderline of statistical significance. It is also very easy to forget that the probability level for significance is largely arbitrary, that a result with a low probability *can* occur, and that statistical tests do not prove anything. Indeed the methods described here are also biased in the sense that antagonism is *assumed* to be competitive unless there is strong evidence (usually $P < 0.05$) to the contrary. Alternative methods are available (Mackay & Wheeler, 1974) which provide estimates of K_I whether antagonism is competitive, non-competitive or pseudo-irreversible provided that the antagonism is selective for the receptor being studied. These latter methods however involve more work initially in setting up the computer programs.

November 28, 1977

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