Br. J. Pharmacol. (1988), **94**, 693–698

An examination of experimental design in relation to receptor binding assays

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1 Real and computer-simulated data were used to examine the efficiency of designs for receptor binding assays.

2 Initially, several different concentrations of [^{3}H]-ketanserin were used in receptor binding studies, using membrane preparations from rat cerebral cortex to establish the form of the binding curves and to investigate the relationship between the variance of the binding measurements and their means.

3 The data showed that the specific binding could be modelled by a simple rectangular hyperbola, and were consistent with the assumption that the binding measurements have a constant coefficient of variation (0.1).

4 Computer-simulated data, with a coefficient of variation of 0.1, were then used to look at the precision of estimates of K_D and B_{max} obtained through the use of assay designs based on replicate incubations at two radioligand concentrations, or through the use of a geometric sequence of 5–7 radioligand concentrations. In each case, the influence of varying amounts (3.8–50%) of non-specific binding at K_D on the precision of these estimates was monitored.

5 The results (a) illustrate the problems which arise in the analysis of receptor binding data when there are relatively high amounts of non-specific binding in combination with a constant coefficient of variation, (b) calculate the errors involved and (c) quantitate the relative merits of 2, 5, 6 and 7 point saturation curves in the estimation of B_{max} and K_D.

Introduction

Despite the existence of an extensive literature on receptors and on methods of analysing data obtained from ligand-receptor binding studies (Klotz, 1982; Marangos *et al.*, 1984; Munson, 1984; Dunn, 1985), relatively little attention has been paid to the problem of selection of the best radioligand concentrations to be used in receptor assays (Klotz, 1982). In the present context, we refer to the word 'best' as meaning the combination of radioligand concentrations that will (for a fixed number of data points) provide the most precise estimates of B_{max} (receptor number) and K_D (affinity of ligand for receptor). In practice, it may not always be possible or even desirable to use the statistically optimal design, but a knowledge of the effects of changes in assay design on the precision of estimates of receptor number should enable the laboratory worker to select an assay design that is efficient in terms of both the statistical properties of the results and the labour and skill needed to carry out the assay.

In receptor studies which use 'binding' assays, it is customary to obtain measurements of specific (saturable) binding of the radioligand to its appropriate receptor by subtracting 'non-specific' binding from total binding at several different concentrations of radioligand. Non-specific binding is measured in samples in which high concentrations of unlabelled ligand are present in the assay tube, on the assumption that the unlabelled ligand will displace specifically-bound radioligand but not that bound to other components of the cell or tissue preparation (i.e. within the range of the assay, the number of non-specific binding sites is effectively infinite). Typically, an arithmetic or geometric sequence of radioligand concentrations is used (in duplicate or triplicate incubations) and the measured amount of specific

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binding fitted to a simple rectangular hyperbola. If pilot studies using a wide range of radioligand concentrations have convincingly validated the use of a simple hyperbolic binding curve, then it is possible to show that the most efficient assay design involves the use of replicate incubations at only two radioligand concentrations. Duggleby (1979) and Currie (1982) for example, have shown that, if it is assumed that measurement errors have a constant variance, then the optimal design is obtained through the use of the lower concentration close to K_D and the upper one as high as possible.

The work of Duggleby (1979) and Currie (1982), however, rests on a statistical model that is unlikely to be valid for most receptor assays. Their model is equivalent to the following:

$$B_i = \frac{B_{max} \cdot F_i}{K_D + F_i} + e_i \qquad (1)$$

where F_i and B_i are the observed concentrations of free and bound radioligand respectively, and B_{max} and K_D are the two unknown parameters corresponding to maximum binding and the free radioligand concentration at half-maximal binding. Measurement error is represented by e_i. A more realistic statistical model for a receptor assay has the following form.

$$B_i = \left(\frac{B_{max} \cdot F_i \cdot X_i}{K_D + F_i} + C \cdot F_i\right)(1 + e_i) \qquad (2)$$

In this equation, X_i is an indicator or 'dummy' variable that has a value of 1 when total binding is being measured and 0 when non-speicific binding alone is being measured. The other new term (CF_i) represents non-specific binding, C being the slope of a straight line describing the relationship between free radioligand concentration and specific binding. Finally, note that the error term, e_i, has been incorporated into the model so that if it has a fixed variance (say, σ^2) then the observations will have a constant coefficient of variation; i.e. the standard error of the measurements is proportional to the expected amount of binding. In both models the measurement errors are assumed to be independent of each other and of the other components in the model.

The aim of the work presented in this paper was two fold. Firstly, to use data from pilot studies to test the applicablity of equation (2) in an assay of 5-hydroxytryptamine$_2$ (5-HT$_2$)-receptors in the rat cerebral cortex (using [^{3}H]-ketanserin; Leysen *et al.*, 1982; Koshikawa *et al.*, 1985). Secondly, to use estimates of the coefficient of variation of bound radioligand measurements obtained from these pilot studies to investigate the efficiency of various assay designs using computer-simulated binding assays.

Methods

Receptor assays

Briefly, rat cerebral cortices were homogenized with a Silverson homogenizer for 15 s in 0.05 M Tris HCl buffer (pH = 7.7) at 4°C, and the homogenate then centrifuged at 48,200 *g* for 15 min at the same temperature. The resulting pellet was washed and centrifuged twice in Tris buffer and finally resuspended in 100 volumes of buffer. The samples were incubated with [^{3}H]-ketanserin (0.1–1.5 nM) in a final volume of 0.5 ml for 15 min at 37°C. Non-specific binding was defined using methysergide (1 μM). After incubation, samples were filtered and washed twice (2 × 5 ml) through Whatman GF/B filters which were then counted in a scintillation counter (Leysen *et al.*, 1982; Koshikawa *et al.*, 1985).

Computer simulations

Equation (2) was assumed to describe binding of radioligand in a receptor assay. Hypothetical data were generated through the use of programmes (MACROS) written using the computer package GLIM (1985). In all cases, both K_D and B_{max} were equal to 1. The choice of equal values for K_D and B_{max} for the computer simulations is completely arbitrary and does not influence the results; it is merely a way of defining the scales of measurement for these parameters. In a real assay, however, one would expect the investigator to choose concentrations of tissue such that the basic assumptions concerning ligand-receptor binding still hold. In the simulations for the present paper, the error terms (e_i's) were independently and identically normally distributed with a mean of 0 and a variance of 0.01 (equivalent to a standard deviation of 0.1). This value corresponds to the coefficient of variation of 0.1 which was found in the pilot studies of receptor binding (see Results). After selection of the appropriate values for F_i, total and non-specific binding were separately generated and specific binding then calculated from the difference between these two. Each simulated assay comprised at least ten (F_i, S_i) pairs, where S_i is the specifically-bound radioligand corresponding to the free radioligand concentration (F_i). GLIM was then used to analyse each of the simulated assays to provide estimates of both K_D and B_{max}. (GLIM is used to fit data to a rectangular hyperbola through the choice of a reciprocal link function and, in this case, gamma distributed errors were specified (see Dunn, 1985).) For each choice of assay design, 250 sets of simulated data were generated and analysed. The standard deviations of the 250 estimates of K_D and B_{max} were then calculated as indicators of the

design's efficiency. The GLIM programme used in these simulation studies is available on request.

Results

Data from binding assays using [^3H]-ketanserin and rat brain cortical membranes

The results of many (>10) receptor binding assays, using six different concentrations of free radioligand ([^{3}H]-ketanserin), indicated that specific binding could be modelled by a simple rectangular hyperbola and in addition, that non-specific binding could be approximated by a straight line. Using rat cerebral cortical membranes and [^{3}H]-ketanserin, we found that the K_D for the binding was approximately 0.45 nM and at this point, approximately 30% of the total binding of radioligand was non-specific (that is, non-displaceable by unlabelled high concentrations (1 μM) of ligand (Koshikawa *et al.*, 1985).

The relationship between the variance of the observations and their mean was of interest. Figure 1 summarizes the results of at least 10 receptor binding assays in which there were six concentrations of [^{3}H]-ketanserin. Each point represents the result of triplicate incubations at a particular radioligand concentration and thus there are 12 points per assay (i.e. 6 total and 6 non-specific). The logarithm of the standard deviation of the three measures of binding is plotted against the logarithm of the corresponding mean. The units of the logarithms are the c.p.m. from the binding assay: however, the slope of the line is not affected by the units used as long as they are common to both axes. The rationale behind this plot is the following. Let the standard deviation (σ) be related to the mean (μ) by the following model:

$$\sigma = \alpha\mu^{\beta} \tag{3}$$

where α and β are unknown constants. Then if logarithms (here to base 10) are taken of both sides:

$$\log(\sigma) = \log(\alpha) + \beta \log(\mu) \tag{4}$$

The slope of the log-log plot is β and the intercept on the vertical axis is log (α). There are several statistical problems in fitting a straight line to the data in Figure 1 (see Finney & Phillips, 1977), but it is clear that the standard deviation is dependent on the mean. The data appear to be consistent with the assumption that $\beta = 1$ and log (α) $= -1$, so that

$$\sigma = \frac{\mu}{10} \tag{5}$$

To summarize, the data are consistent with the observed amount of [^{3}H]-ketanserin binding in the rat cortex preparations having a coefficient of variation of 0.1. They are not consistent with the assumption of constant variance for the measurement errors in the model represented by equation (1).

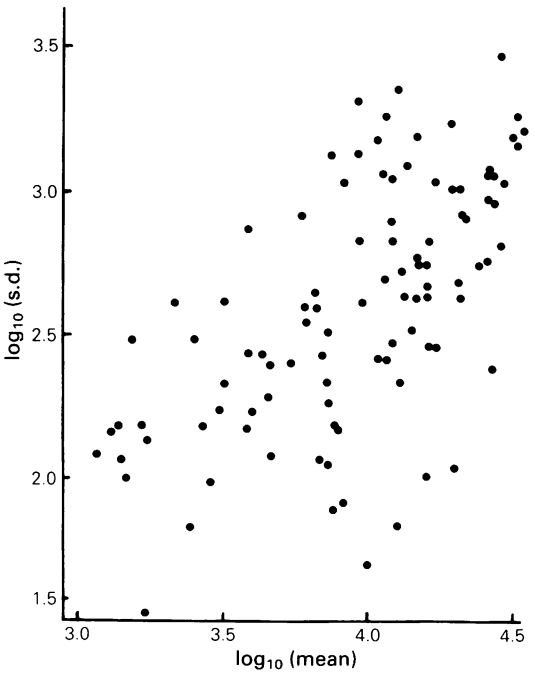

Figure 1 Relationship between the logarithm of the standard deviation and logarithm of the mean for [^{3}H]-ketanserin binding to rat cerebral cortex preparations. Each point is based on three incubations. The log values are calculated from the c.p.m. of [^{3}H]-ketanserin bound. The data are from at least 10 receptor binding assays using 6 concentrations of [^{3}H]-ketanserin, i.e. each assay provides twelve sets of triplicates (6 total and 6 non-specific).

Computer simulated assays

Four different sets of simulated data were generated and analysed. These corresponded to fixing the slope of the non-specific binding curve line (the constant C) to 0.02, 0.1 and 0.25 and 0.5, respectively. These slopes correspond to a characteristic percentage of non-specific binding at K_D (that is, 3.8%, 16.7%, 30% and 50%, respectively). The two extremes might be considered to represent (a) ideal conditions and (b) an assay that is almost non-viable: the reasons for this will become apparent from the results of the computer simulations.

For each of the four sets of simulations, several alternative assay designs were employed. These were not used to demonstrate which design is the 'best',

Table 1 Standard deviations ($\times 10^2$) of estimates of B_{max} and K_D obtained from 250 computer-simulated assays using two-point designs (five incubations at each of the two concentrations)*

	Non-specific binding constant, C (% non-specific binding at K_D)											
	0.02 (3.8%)			0.10 (16.7%)			0.25 (33%)			0.50 (50%)		
	Lower radioligand concentration ($\times K_D$)											
	0.25	0.50	1.0	0.25	0.50	1.0	0.25	0.50	1.0	0.25	0.5	1.0
Upper radioligand concentration ($\times K_D$)												
4	6.78	7.37	8.95	9.58	10.37	12.53	15.46	16.71	20.35	23.39	27.75	36.14
	11.49	14.88	23.56	14.72	19.23	31.14	21.94	28.95	48.62	35.00	47.23	89.29
8	6.25	6.48	7.00	10.94	11.32	12.17	20.21	20.90	22.52	33.72	35.14	38.70
	10.35	12.88	18.55	15.48	19.36	28.16	26.65	33.40	49.08	43.86	55.67	85.10
16	6.70	6.81	7.04	15.41	15.65	16.15	29.70	30.23	31.37	50.19	51.52	54.55
	10.43	12.75	17.66	20.24	24.76	34.37	37.69	46.24	64.67	63.77	79.18	114.2

* The upper values of each pair of estimates is the standard deviation of B_{max}, the lower value is that of K_D.

but merely to illustrate the likely behaviour of real assays using fairly realistic assumptions concerning the generation of the data. The designs ranged from a choice of only two radioligand concentrations (the lower one being $0.25\,K_D$, $0.5\,K_D$ or K_D and the upper one being $4\,K_D$, $8\,K_D$ or $16\,K_D$ – giving 9 permutations) to a geometric sequence of 5, 6 or 7 concentrations. In most cases, the design points were replicated to give the required 10 observations for each assay. When 6 or 7 concentrations were used there were 12 or 14 observations per assay, respectively.

Table 1 shows the results when only two radioligand concentrations are used and Table 2 shows the corresponding results when geometric sequences of concentrations are used. In Table 1, it can be seen that where there is very little non-specific binding (3.8% at K_D), the best results were achieved when the two concentrations of radioligand were $0.25\,K_D$ and $8\,K_D$. Under these experimental conditions, the standard deviation of the B_{max} is not particularly sensitive to the radioligand concentrations chosen. The standard deviation of K_D, however, is increased as the lower of the two concentrations is raised to K_D.

With higher amounts of non-specific binding, the precision of the estimates of both B_{max} and K_D decreases (i.e. their standard deviations increase). The best results are obtained when the lower radioligand concentration is $0.25\,K_D$, but again, the standard deviation of B_{max} is not sensitive to the choice of the lower radioligand concentration. For a given choice of the lower concentration, it can be seen that the more precise estimates of both B_{max} and K_D are obtained when the upper concentration of radioligand is $4\,K_D$. The choice of $8\,K_D$ for the higher concentration does not substantially reduce the precision of the estimates, but the precision is always lower than when $4\,K_D$ is used. In simulations where non-specific binding is 50% of the total at K_D, it can be seen that the standard deviations of the estimates are too high for the estimates to be of much use (particularly the estimates of K_D).

Table 2 shows the standard deviations of parameter estimates of B_{max} and K_D from computer simulations of assays using geometric sequences of 5, 6 or 7 duplicate concentrations of ligand and, as above, with four different amounts of non-specific binding. The precision of the estimates of B_{max} and K_D clearly decreases with increasing amounts of non-specific binding. When there is a substantial amount of non-specific binding, it is clearly desirable not to use high radioligand concentrations in these assays. What, in practice, is regarded as a substantial amount of non-specific binding, will depend on the coefficient of variation of the binding measurements.

Discussion

The first finding of this study is that the measurements of [^{3}H]-ketanserin binding to rat cortex preparations do not have a constant variance (or equivalently a constant standard deviation) but a constant coefficient of variation, that is, their standard deviation is directly proportional to the amount of radioligand binding being measured. The constant of proportionality in the present assays is approximately 0.1. This finding is consistent with similar data from radioimmunoassays (Finney & Phillips, 1977) and is also similar to results obtained from kinetic studies of enzymatically catalysed reactions (Cornish-Bowden, 1979). In an ideal assay (i.e. no non-specific binding), this finding would not be of great significance. In reality, it is important for three reasons. First, it provides a proven explanation of

Table 2 Standard deviations ($\times 10^2$) of estimates of B_{max} and K_D obtained from 250 computer-simulated assays using designs based on geometric sequences of radioligand concentrations (duplicate incubations at each concentration)*

Concentrations ($\times K_D$)	*Non-specific binding constant, C* (% non-specific binding at K_D)			
	0.02 (3.8%)	0.10 (16.7%)	0.25 (33%)	0.50 (50%)
0.125, 0.25, 0.50, 1, 2	11.03	13.33	19.23	28.57
	16.41	19.44	27.06	40.89
0.25, 0.50, 1, 2, 4	8.48	11.61	18.21	29.82
	15.24	19.40	30.08	45.98
0.50, 1, 2, 4, 8	7.49	12.09	22.13	38.43
	17.47	24.96	44.95	75.76
1, 2, 4, 8, 16	7.13	14.86	27.34	56.15
	22.49	41.72	74.17	164.00
0.125, 0.25, 0.50, 1, 2, 4	7.74	9.92	15.84	25.60
(6 concentrations)	12.77	15.88	23.72	37.93
0.25, 0.50, 1, 2, 4, 8	6.28	10.30	17.50	33.16
(6 concentrations)	13.10	18.42	30.67	56.64
0.125, 0.25, 0.50, 1, 2, 4, 8	6.31	9.73	16.85	28.66
(7 concentrations)	10.55	15.09	24.96	41.53

* The upper value of each pair of estimates is the standard deviation of B_{max}, the lower value is that of K_D.

why one cannot reliably measure receptor concentrations in the presence of large amounts of non-specific binding. In Table 1, for example, it can be seen how dramatically the standard deviations of both B_{max} and K_D increase as one moves to the right of the table (i.e. as the non-specific binding constant increases). When 50% of the binding at K_D is non-specific, the estimates (for K_D in particular) are likely to be almost worthless as their standard deviations will be much too large. The second implication of this finding is for the way in which data are fitted to binding curves to provide estimates of K_D and B_{max}. In the present simulation studies, measurements were assumed to be distributed following a gamma distribution (they have to be greater than or equal to zero with a standard deviation proportional to their mean) and the estimates obtained through the use of maximum likelihood. Although this assumption is not strictly valid for modelling specific binding alone, in the case of the two-point designs the assumptions concerning the distribution of the errors (assuming that there are no outliers) do not influence the values of the estimates. However, in the other designs such as those shown in Table 2, they can. If normally-distributed errors with a constant variance are assumed for the binding measurements, then the resulting estimates are far less precise than those given in Table 2, particularly when there is a high proportion of non-specific binding. If assays for other types of receptor behave similarly to the present example, then the use of ordinary least squares to fit binding data to theoretical curves is not the most efficient use of the data. Maximum likelihood or weighted least squares estimators should be used instead.

The third, and perhaps the most interesting, implication of the findings of the present study concerns the choice of a sensible assay design. The work of Duggleby (1979) and Currie (1982) does not provide an optimal design for a receptor assay. For a two-point assay the precision of the estimates increases as the lower radioligand concentration is lowered. The precision of the estimates also increases with increasing upper radioligand concentration if there is little or no non-speicific binding, but for any realistic level of non-specific binding it will deteriorate dramatically above approximately $4K_D$. When high radioligand concentrations are used, the data will be completely dominated by non-specific binding. With non-specific binding at levels shown on the right of Tables 1 and 2, it will not be uncommon, for example, to get negative estimates for the amount of specific binding in incubations using high radioligand concentrations (in the above computer simulations a programme line was added to prevent the occurrence of negative levels of specific binding by equating them with zero when they occurred). If the variance of the binding measurements were constant, then this phenomenon would not occur and the relative amount of non-specific binding would not determine whether a particular assay or assay design is a practical proposition.

The above discussion on assay design does not simply apply to the two-point assays. If a cautious worker chooses to use a wide range of radioligand concentrations to test the applicability of a model

such as equation (3), then he or she should still be aware of the potential problems caused by the use of relatively high radioligand concentrations. This is clearly illustrated in Table 2. On the left (very little non-specific binding) the inclusion of high radioligand concentrations increases the precision of estimates of B_{max} (but not necessarily of K_D). On the right, however, high radioligand concentrations appear to be detrimental to the precision of both.

The choice of an assay design is clearly dependent on several considerations. Designs that are optimal in terms of the statistical properties of the resulting estimators will usually preclude any testing of underlying assumptions concerning the characteristics of ligand binding. If the optimal two-concentration design is chosen for the measurement of receptor concentrations, then it is vital that any assumptions are tested thoroughly in preliminary studies and perhaps at times throughout the main investigation itself as a quality control exercise. In many cases, the investigator might decide that the costs of using the statistically most efficient design outweigh the advantages. In choosing an assay design, however, one should, at least, be aware of the statistical characteristics of ligand-receptor interactions and of the statistical properties of potential receptor assay designs.

Given the obvious pitfalls encountered in using the simple design involving the use of two radioligand concentrations, why should an investigator choose to use it? Possible reasons will be illustrated by reference to the present authors' attempts to demonstrate circadian rhythms in neurotransmitter receptors in different areas of the rat brain (Koshikawa *et al.*, 1988). A single experiment may involve the dissection of brains obtained from up to 100 rats. Animals need to be sampled every 4 h for at least 3 consecutive days to have any chance of demonstrating a reproducible 24 h rhythm. If each animal provides homogenates from 5 separate areas of the brain this will lead to at least 500 receptor assays per experiment. In this type of experiment an efficient receptor assay is needed for a number of reasons. These are (a) the cost of radioactively-labelled ligands, (b) tissue availability – some of the neuronal tissues studies, are obtained from very small areas of the brain such as the hypothalamus and pineal gland, and (c) reduction in the amount of labour involved and hence reduced risks of serious pipetting errors.

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(Received November 7, 1987
Accepted February 23, 1988)