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A PRACTICAL MODEL FOR STEROID HORMONE RADIOIMMUNOASSAYS

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SUMMARY

The application of one plausible restriction, namely that the antiserum be effectively saturated with steroid under all assay conditions, is shown to lead to a simple hyperbolic binding function for the description of steroid hormone radioimmunoassay standard curves. This model is shown to have distinct advantages over conventional models; (a) data evaluation is greatly simplified; (b) further analysis of the model enables the precision and sensitivity of an assay to be simply determined for any assay conditions and hence provides direct criteria for the establishment of optimised assays. Experimental proof of the validity of the model is presented and examples of its application to a variety of steroid hormones are given.

INTRODUCTION

Since its introduction [1, 2] the technique of radioimmunoassay (RIA) has been developed into a powerful tool, applicable to a wide range of biological substance whose nature or low *in vivo* concentrations make them difficult to analyse by any other means. Naturally the theoretical aspects of the technique have received much attention [3–5] but hitherto such mathematical treatments have been complex and open to dispute [6] because the application of the law of mass action in the general case (i.e. without the imposition of restrictions) gives rise to unwieldy expressions which, for the non-mathematically inclined at least, are difficult to manipulate and interpret. The purpose of this paper is to show that, for homologous assays, i.e. those in which the labelled and unlabelled ligand are chemically and immunologically indistinguishable (as is the case in steroid hormone assays), the imposition of one simple, plausible restriction reduces the law of mass action functions to an expression which is not only simple to manipulate and interpret but also is of practical significance in that it predicts optimum experimental conditions which are rather different from those commonly employed at present.

Derivation of the model

Let us impose on our RIA technique the restriction that the antibody be effectively saturated (i.e. >99% of available sites occupied) with ligand (labelled or unlabelled) under all assay conditions. It therefore follows that, for homologous assays, the proportion of sites occupied by labelled ligand is the same as the overall (i.e. bound and free) proportion of labelled ligand in the assay mixture, i.e.

$$\frac{[AL]}{[AL] + [AX]} = \frac{[L_t]}{[L_t] + [X_t]}$$

but, when the antibody is saturated with ligand, $[AL] + [AX] = [A_t]$ hence

$$[AL] = [A_t].\frac{[L_t]}{[L_t] + [X_t]} \qquad (1)$$

where

- $[A_t]$ = total concentration of ligand binding sites (occupied and unoccupied);
- $[AL]$, $[AX]$ = concentrations of sites occupied by ligands L (labelled) and X (unlabelled) respectively;
- $[L_t]$, $[X_t]$ = total concentrations (bound and free) of ligands L and X.

Equation 1 can be derived formally using the law of mass action as shown in the appendix.

However, in order to measure $[AL]$ it is necessary to separate the bound and free ligands—a process which is rarely completely efficient. It is our experience that, in the commonly employed activated charcoal/Dextran procedure, the residual free ligand after absorption is a constant fraction of the total free ligand, i.e.

$$R = K \cdot F$$

where

- R = residual free ligand (after absorption);
- F = free ligand before absorption. For the labelled ligand, $F = [L_t] - [AL]$;
- K = constant.

Since a proportion of the residual free ligand will be labelled it will be measured along with the bound ligand, i.e.

$$Lm = [AL] + [Lr]$$

where

Lm = measured concentration (bound and residual) of labelled ligand;

$[Lr]$ = concentration of residual free labelled ligand = $K([L_t] - [AL])$.

Thus

$$Lm = [AL](1 - K) + K[L_t]$$

$$= [A_t]\cdot(1 - K)\cdot\frac{[L_t]}{[L_t] + [X_t]} + K[L_t]$$

Under the normal assay conditions of constant antibody concentration, labelled ligand concentration and charcoal/Dextran concentration, $[A_t]$, $[L_t]$ and K are all constants and the function is more conveniently written.

$$Lm = \frac{B}{L + X} + C \qquad (2)$$

where

$$B = [A_t](1 - K)[L_t]$$

$$C = K[L_t]$$

L and X substitute for $[L_t]$ and $[X_t]$

Derivation of the model requires that the antibody be saturated with ligand under all assay conditions. The most unfavourable case is that corresponding to zero concentration of unlabelled ligand i.e. the concentration of labelled ligand alone must be sufficient to saturate the antibody. The conditions for fulfilment of this requirement are readily established by plotting not the conventional dilution curve but the concentration curve (e.g. Fig. 1). The linear region as concentration approaches zero represents the range of antibody concentrations for which the requirement is met for the given amount of labelled ligand. It is important to note that a consequence of this condition is that heterogeneity in the antibody (i.e. sites having different affinities) has no effect on the binding function. However, it is essential that the assay system be at equilibrium and in this respect the kinetic aspects of RIA [7] are of crucial importance.

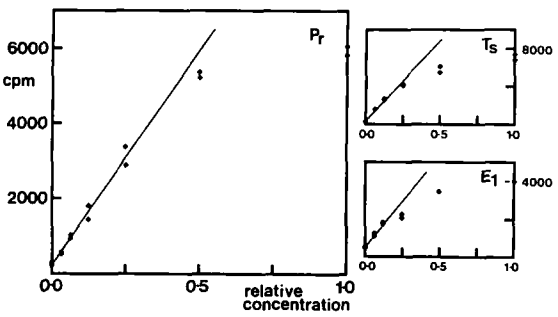

Fig. 1. Concentration curves. The concentration curves are constructed by determining the amount of bound labelled ligand (ordinate) at various antiserum concentrations (the *total* concentration, i.e. bound and free, of labelled ligand is constant). Since the absolute concentration of antiserum is not known, the abscissa is scaled in relative concentration (the highest concentration, i.e. stock antiserum solution, being unity). The linear region at low concentrations is the range corresponding to effective saturation of the antiserum by the labelled ligand. The three curves illustrated are for anti-progesterone-11αBSA-serum (*Pr*), anti-testosterone-7αBSA-serum (T_s) and anti-estrone-6,thyroglobulin-serum (E_1). Note that the anti-estrone-serum has a high intercept on the *y*-axis indicating that the charcoal/Dextran is relatively inefficient at absorbing free estrone.

Validation of the model. Equation 2 is a hyperbolic function having asymptotes of $X = -L$ and $Lm = C$. It has long been recognised that a graph of Lm vs X, as produced from RIA standard curve data, is hyperbolic in appearance. This has prompted Kibby (personal communication) and Walters [8] on purely empirical grounds, to employ hyperbolic functions as an alternative to the conventional logit transform for the computerised evaluation of RIA data and in both cases superior descriptions of the data were reported. However, Walters employed the general 4-parameter hyperbolic function.

$$Lm = \frac{b}{(r + X)^s} + a$$

(a, b, r and s are empirical constants)

whereas Kibby employed a 3-parameter function

$$Lm = \frac{K_3}{K_1 + X} + K_2$$

(K_1, K_2 and K_3 are empirical constants)

which is a special case of the 4-parameter function in which $s = 1$ and is identical in form to equation 2. When the 4-parameter function is fitted to RIA data the parameter s is *not* usually found to be close to unity (Heap, private communication) suggesting that it is of some significance. However, the parameters r and s are such that a change in one can, to a large extent, be compensated by a change in the other so that the fit may be almost as good even when s is constrained to be unity.

Furthermore, Walters (loc. cit.) reports that, when using the 4-parameter function, "the convergence process....failed with some assay data due to steadily increasing values of r and s" so that no satisfactory fit was obtained. Several sets of such non-convergent data have been made available to us and in each case, satisfactory standard curves were obtained (Fig. 2) when the 3-parameter function was employed. Thus, in view of the fact that derivation of equation 2 provides no explanation for the parameter s it would appear to be superfluous.

However, the ability to "fit" experimental data, though a necessary condition for validating a model, is not sufficient in itself and we have therefore sought additional experimental evidence. The derivation of equation 2 predicts that all three parameters, B, C and L, should vary linearly with the amount of labelled ligand employed in the assay. That this is so is illustrated in Fig. 3.

It is believed that the ability of the model to "fit" the data and to predict the effects of changes in exper-

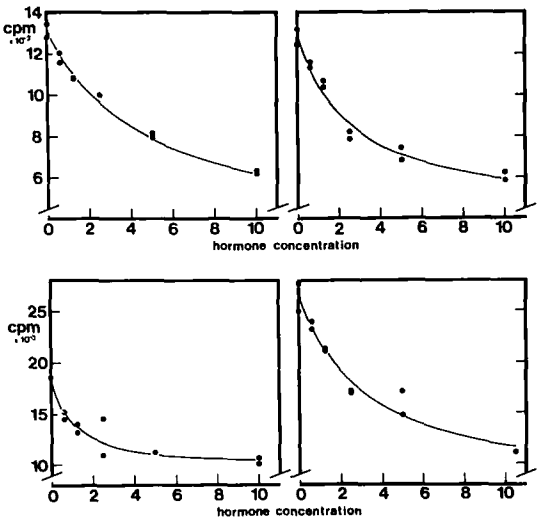

Fig. 2. "Non-convergent" standard curve data. For practical reasons [8] the hormone concentration is scaled to be 0–10. In all cases illustrated the scaling is 0.08 ng/ml i.e. 10 ≡ 0.8 ng/ml.

When fitting the 4-parameter function

$$Lm = \frac{b}{(r + X)^s} + a$$

to the data illustrated the least-square curve-fitting process [8] failed to converge to a satisfactory fit. However, when the 3-parameter function

$$Lm = \frac{K3}{K_1 + X} + K_2$$

was used the least-squares curve fitting converged and produced the best-fit lines as illustrated. Thus, the 3-parameter function is preferable to the 4-parameter function since the additional exponential parameter is not only unnecessary for an acceptable "fit" to the data (and in some cases even inhibits convergence) but also has no apparent role in the derivation of the binding function.

imental conditions on the values of the parameters together constitute reasonable grounds for accepting the model as valid.

Since the parameters of the model (equation 2) can be explicitly related to the experimental conditions employed in an assay, it can be analysed to predict the properties of an assay system under any given conditions.

Rearrangement of equation 2 gives

$$X = \frac{B}{Lm - C} - L \qquad (3)$$

enabling X to be calculated from Lm once B, L and C are known. The variance in X, $V(X)$ at any point can be calculated using the approximation (for non-linear functions)

$$V(X) \simeq \left(\frac{\partial X}{\partial Lm}\right)^2 \cdot V(Lm)$$

where $V(Lm)$ is the variance in Lm,

$$\text{i.e. } V(X) \simeq \left\{-\frac{(L + X)^2}{B}\right\}^2 \cdot V(Lm)$$

but it is the coefficient of variation of X, $CV(X) = \sqrt{V(X)}/X$ which is of greater importance for determining the working range of an assay,

$$CV(X) = \frac{L}{B}\frac{(1 + n)^2}{n} \cdot \sqrt{V(Lm)} \qquad (4)$$

where

$$n = X/L.$$

In the conventional assay procedure the variance in Lm is a combination of two factors, (i) experimental errors (e.g. pipetting errors)

$$V(Lm)_e = (Lm \cdot E)^2$$

where E is the coefficient of variation of the experimental errors and (ii) counting errors (i.e. errors inherent in the random nature of the radioactive decay process)

$$V(Lm)_c = (Lm)^2/T$$

where T is the total count.

Thus, the total variance in Lm is

$$V(Lm)_t = V(Lm)_e + V(Lm)_c = (Lm)^2 (E^2 + I/T) \qquad (5)$$

hence, the overall coefficient of variation of X is

$$CV(X) = \frac{L}{B} \cdot \frac{(1 + n)^2}{n} \cdot Lm \cdot \sqrt{E^2 + I/T} \qquad (6)$$

Substitution of Lm by equation 2, writing $Lo = Lm$ when $X = 0$ and $r = C/Lo$ leads ultimately to

$$CV(X) = \frac{1 + n}{n} \cdot \frac{1 + nr}{1 - r} \cdot \sqrt{E^2 + I/T} \qquad (7)$$

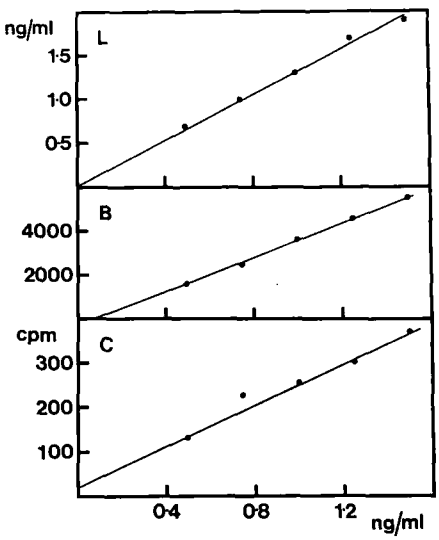

Fig. 3. The effect of labelled ligand concentration on the parameters of the binding function. The derivation of the binding function, equation 2, predicts that the parameters B, C and L (ordinates) should vary linearly with the amount of labelled ligand employed (abscissa) at constant antiserum concentration. This is found to be the case as illustrated in this example using an anti-estriol-6BSA-serum. The parameters are obtained directly as described in the text, *not* by least-squares curve-fitting.

The term

$$\frac{1+n}{n}\cdot\frac{1+nr}{1-r},$$

illustrated in Fig. 4, is the error factor, i.e. the factor by which the coefficient of variation of Lm must be multiplied to give the resultant coefficient of variation of X.

Although the experimental coefficient of variation, E, can be regarded as constant, the counting coefficient of variation, I/T, is clearly only constant if all samples are counted until a predetermined number of disintegrations are observed. If, as is more common, samples are counted for a predetermined time then $T \propto Lm$ (i.e. the counting error increases as n increases) but, *provided* that $I/T \ll E^2$, this has little effect on the coefficient of variation of X.

It can be seen that the parameter r is of crucial importance in determining the error factor, particularly at higher n-values. Clearly, for maximum precision and the widest working range, r must be kept as low as possible by reducing the value of C (i.e. increasing the efficiency of the sequestration of free from bound steroid) and by increasing the value of Lo (i.e. working at the highest antiserum concentration consistent with the requirement that the antiserum is always saturated).

At first sight it appears that this model predicts

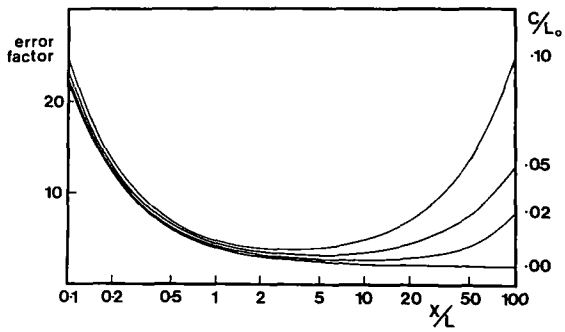

Fig. 4. Error functions. The relationship between coefficients of the variation of the procedural errors and the resultant coefficient of variation in the measured amount of steroid, X, is

$$CV(X) = \frac{1+n}{n}\cdot\frac{1+nr}{1-r}\cdot\sqrt{E^2 + I/T} \quad \text{(equation 7)}$$

where n is the ratio unlabelled to labelled steroid, X/L, and r is the ratio of residual to bound ligand, C/Lo. The values of the error factor.

$$\frac{1+n}{n}\cdot\frac{1+nr}{1-r}$$

as functions of n at various values of r are illustrated. Typical values for r are: Anti-estrone-6,thyroglobulin-serum 0.20; Anti-estradiol-6BSA-serum 0.02; Anti-estradiol-17βBSA-serum 0.08; Anti-estriol-6BSA-serum 0.06; Anti-progesterone-11αBSA-serum 0.06; Anti-testosterone-7αBSA-serum 0.06. All antisera supplied through Miles Laboratories Ltd., Slough, England.

that the working range can be reduced *ad infinitum* simply by reducing L, the amount of labelled steroid employed, since the error factor contains only the X/L ratio, n, not the absolute value of L. This of course, is not actually the case. In practice the lowest working range is determined by (a) the specific activity of the labelled ligand, which limits the lowest practical amount of labelled steroid before the counting errors become unacceptably high and/or (b) the afinity constant of the antiserum which limits the lowest practical concentration of antiserum before the parameter r becomes unacceptably high.

DISCUSSION

The simplified model is, of course, merely a special case of the general models [3, 7] and thus has the same general properties (e.g. the conditions for maximum precision and working range) although there are certain differences arising from its application (e.g. the antibody concentration is chosen to ensure that it is always saturated rather than to give a bound/free ligand ratio of 1). Nevertheless, the simplified model offers several substantial practical advantages over the general models: (i) it provides simple, explicit criteria for the direct selection of appropriate assay conditions; (ii) data evaluation (both computation of the standard curve and calculation of the ligand concentration in unknown samples) is extremely simple; empirical (e.g. logit) transforms are eliminated and, if computer facilities are not available, a desk-top calculator is sufficient, (iii) the explicit relationship between the parameters of the model and the experimental conditions enables the performance of the assay to be closely monitored.

(i) The working range of an assay is the range of n-values for which the coefficient of variation is less than the maximum acceptable value. It can be seen from equation 7 that where two n-values, n_1 and n_2, have the same coefficient of variation (e.g. the lower and upper ends of the working range) they are related by $n_1 \cdot n_2 = 1/r$ (E^2 assumed constant; $1/T$ assumed constant or insignificant).

It is clear from Fig. 4 that, under all conditions, the coefficient of variation rises very rapidly when $n < 1$. Thus, as a convenient "rule of thumb," the lower end of the working range coincides with the amount of labelled ligand employed, i.e. $n = 1$, and hence the upper end of the working range is simply given by $n_2 = 1/r$. Setting up an assay with known characteristics consists, therefore, of three simple steps; (1) select the amount of labelled ligand equal to the lower end of the desired working range; (2) construct a concentration curve (as described earlier) to determine the appropriate antibody concentration; (3) calculate the value of r from the bound labelled ligand (zero antiserum concentration) to determine the upper end of the working range (remembering that this may be reduced if the counting errors rise to significant levels as Lm decreases).

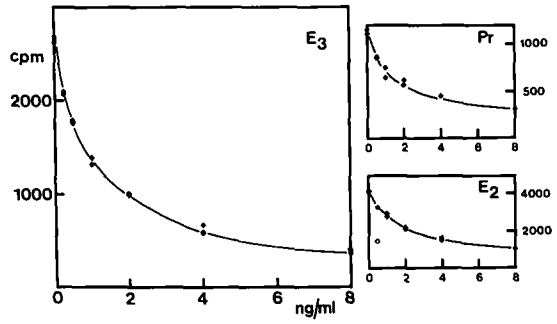

Fig. 5. Standard curves, ◆ experimental data. —— the value of the function

$$Lm = \frac{B}{L + X} + C$$

where the parameters B, C and L have been obtained directly as described in the text, i.e. least-squares curve-fitting has *NOT* been employed. The curves illustrated are for anti-estriol-6BSA-serum (E_3), anti-progesterone-11αBSA-serum (Pr) and anti-estradiol-17βBSA-serum (E_2).

(ii) The explicit relationships which exist between the experimental conditions and the parameters of the model makes the computation of standard curves and subsequent calculations of unknowns so simple that they can readily be done on a desk-top calculator.

Consider equation 2:

When $[A_t] = 0$ (i.e. antibody omitted and buffer substituted) $C = Lm$. Thus inclusion of "no antiserum" controls gives C directly.

In principle. the value of L can be determined from the specific activity of the labelled ligand but it is our experience that this may not be sufficiently reliable so the following approach is employed instead

Let

$$Lo = Lm \text{ when } X = 0$$

$$L_2 = Lm \text{ when } X = L$$

Then

$$Lo = \frac{B}{L} + C \text{ and } L_2 = \frac{B}{2L} + C.$$

Thus, on rearrangement $L_2 = (Lo + C)/2$, i.e. the value of L is the value of X on the standard curve at which $Lm = L_2$. This method, of course, requires that the standard curve be drawn but it is our opinion that this is good practice and should be done in any case.

Once C and L are known, B is readily determined from $Lo = B/L + C$.

Examples of standard curves calculated in this way are shown in Fig. 5. Of course, the model may also be used as the basis for iterative least-squares curve-fitting in which some or all of the parameters are unconstrained and there are some who would argue that the values of the parameters are of little consequence provided that the "fit" is good. Clearly, those who have the choice will make their own decision; our experience is that, where computational facilities are limited, the approach which has been described is simple to employ and provides acceptable results.

(iii) Radioimmunoassay is a notoriously unpredictable technique, liable to produce spurious results without any apparent reason. In the majority of cases the quality of the standard curve is the only available index of assay performance. The explicit relationships which exist between the experimental conditions and the parameters of the model confer the advantage that aberrant values of the parameters not only provide helpful clues as to the nature of the difficulty but can also indicate a deterioration in assay performance even when the standard curve appears to be satisfactory and there are no other obvious signs of assay malfunction.

Although RIA is a widely used technique, the lack of a simple yet practical model for the assay system has meant that in a large number of cases the establishment and running of assays has been essentially empirical (and may therefore be suboptimal) and the calculation of results complex. The model which has been proposed is not a radical departure from conventional RIA theory, merely a special case limited by the simple restriction which has been imposed. In general, therefore, the properties of the model are consistent with currently held views, but the effect of the restriction is to simplify its applications so that assays can now be *designed* to have the desired working range and data evaluation is reduced to a simple, explicit procedure which requires no elaborate computational facilities. It is these properties which, we believe, will make the model of considerable value to those working with steroid hormone and other homologous radioimmunoassays.

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APPENDIX

Derivation of the binding function, equation 1.

Consider an antibody having j reversible ligand binding sites; the law of mass action functions for the ith site are:

$$E_L i = \frac{[AL]i}{[A]\,[Lf]} \qquad E_X i = \frac{[AX]i}{[A]i\,[Xf]}$$

where

$[Lf]$ and $[Xf]$ are the concentrations of free ligand, labelled and unlabelled respectively,

$[A]i$ is the concentration of the unoccupied ith binding site,

$[AL]i$ and $[AX]i$ are the concentration of the ith binding sites occupied by labelled and unlabelled ligand.

$$[A]i = \frac{[AL]i}{E_L i\,[Lf]} = \frac{[AX]i}{E_X i\,[Xf]}$$

i.e.

$$[Xf]\,[AL]i = [Lf]\cdot\frac{E_L i}{E_X i}\cdot[AX]i$$

When L and X are chemically and immunologically indistinguishable, as is essentially the case with tritium-labelled steroid hormones, $E_L i = E_X i$, hence, summing for all j sites

$$[Xf]\sum_{i=1}^{i=j}[AL]i = [Lf]\sum_{i=1}^{i=j}[AX]i$$

But

$$[L_t] = [L_f] + \Sigma[AL]i \text{ and } [X_t] = [X_f] + \Sigma[AX]i$$

where $[L_t]$ and $[X_t]$ are the total concentrations (free and bound) of L and X, hence

$$([Xt] - \Sigma[AX]_i)\,\Sigma[AL]i = ([L_t] - \Sigma[AL]i)\,\Sigma[AX]i$$

i.e.

$$[X_t]\cdot\Sigma[AL]i = [L_t]\,\Sigma[AX]i$$

The total (occupied and unoccupied) concentration of binding sites, $[A_t]$, is:

$$[A_t] = \Sigma[AL]_i + \Sigma[AX]i + \Sigma[A]i.$$

However, under conditions such that the antibody is effectively saturated with ligand, $\Sigma[A]i$ is negligible, hence

$$[A_t] \simeq \Sigma[AL]_i + \Sigma[AX]i.$$

Thus

$$[X_t]\,\Sigma[AL]_i \simeq [L_t]\,\{[A_t] - \Sigma[AL]_i\}$$

i.e.

$$\{[X_t] + [L_t]\}\,\Sigma[AL]_i \simeq [L_t]\cdot[A_t]$$

whence

$$\Sigma[AL]_i \simeq [A_t]\cdot\frac{[L_t]}{[X_t] + [L_t]}$$

i.e. Equation 1 in main text.

Q.E.D.