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Mathematical Modelling of Competitive Labelled-Ligand Assay Systems

Theoretical Re-Evaluation of Optimum Assay Conditions and Precision Data for Some Experimentally Established Radioimmunoassay Systems

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Summary: A mathematical theory of competitive labelled-ligand assays was developed with the intention of theoretically re-evaluating the optimal assay conditions and precision data of assay systems established by experiment. Our theory is based upon the assumptions of a simple bimolecular reaction mechanism, homogeneous reactants, as well as kinetically indistinguishable labelled and non-labelled ligands. The general case of two-step (non-equilibrium) assay was considered including the one-step (equilibrium) assay as a special case. The solution of the system of corresponding kinetic differential equations was used to mathematically construct standard curves. Furthermore, intraassay precision profiles and indices as well as detection limits were calculated considering solely the pipetting error, ε, as a source of experimental error. A procedure was outlined to mathematically determine the optimal incubation conditions for any assay system targeted to a given analyte concentration, P, at which the standard deviation of assay results is to be minimized. Estimates of both the content of binding sites and the equilibrium constant, K, of the specific binding agent are necessary, and these can be derived from *Scatchard* plots. For six RIA systems, of which three were one-step and three were two-step assays, experimental assay conditions and precision data were compared with theoretical predictions. Experimentally determined antibody binding site concentrations agreed fairly well with those independently evaluated by mathematical optimization. Mean precision indices, defined as constituting an average over the complete precision profile, were found to be within the theoretically predicted range, i.e. two- to threefold the pipetting error. Detection limits (standard deviation near concentration 0) differed from theoretical values at most by a factor of two in the case of two-step assays and were nearly identical with theoretical values for one-step assays. Generally, they were of the order of εP, approaching a lower limit by the order of εK, when P falls to the order of K. Comparing the advantages of the one-step and two-step technique of competitive labelled ligand assays, the following results were obtained: The one-step method provides a more favourable precision profile, especially a better detection limit, and a higher specificity of analyte recognition. The quantity of reagents needed (specific binding agent as well as labelled ligand) is three to four times lower than in the two-step method. On the other hand, the higher amount of reagents employed for the two-step technique results in a considerably higher measuring signal, which is important where activity of the labelled ligand is low. We conclude that mathematical modelling of labelled-ligand assays should be useful in re-evaluating assay conditions and precision data obtained by experiment. Furthermore, it permits some general assertions concerning the principal limits of assay precision as well as the advantages and disadvantages of different assay protocols.

Introduction

Since the introduction of their basic analytical principle in 1959 (1), radioimmunoassays (RIA), enzyme immunoassays and other related methods, generally named labelled-ligand assays, have found wide application in clinical chemistry and biochemistry. To quantify a certain analyte in a biological fluid, competitive or non-competitive (sandwich) immunoassays

may be used. The latter have been shown to be more sensitive by as much as orders of magnitude (2, 3). Nevertheless, because of their lower technical requirements, competitive assays are at present more common. If a certain range of analyte concentrations is to be assayed, an optimization of assay conditions is needed which allows the determination of these concentrations with maximal precision. For this purpose the following parameters and conditions have to be adjusted:

(1) concentration of specific binding sites

(2) concentration of labelled ligand

(3) duration of incubation steps

(4) the incubation protocol, i.e. labelled and unlabelled ligand are added simultaneously or the labelled ligand is added after a period during which unlabelled ligand is allowed to preincubate with the binding agent (delayed addition of labelled ligand). In the following, these different incubation protocols will be designated as the one-step or two-step method, respectively.

Usually, optimal conditions of incubation are determined by experiment. Several authors (2, 4–9) have calculated optimal conditions of incubation by using mathematical models which describe the assay reactions by kinetic differential equations (two-step assay) or algebraic equations derived from the mass action law (one-step assay). However, the actual determination of the inevitably necessary reaction parameters (kinetic and equilibrium constants) needs empirically optimized assay conditions. Thus the predictive value of mathematical models is restricted to more or less general assertions or to subsequent corrections of experimentally obtained assay conditions. The aim of our study was therefore to calculate optimal assay conditions and precision data, not in advance, but with the purpose of validating them when they have already been obtained by experiment. As an example, this was undertaken for six RIA systems for peptide antigens. In addition, the one-step and two-step incubation techniques were compared with respect to their influence on intensity of the measuring signal and accuracy of assay results.

Methods

Mathematical theory of competitive labelled-ligand assays

To mathematically describe the kinetics of the assay reactions, several strictly simplifying assumptions must be made:

i) There is a simple bimolecular mechanism of the reaction of a specific binding site, Q, with both labelled (P*) and non-labelled ligand (P). Consequently, this means that all binding sites are kinetically homogeneous and bind independently of each other.

ii) Labelled and non-labelled ligand are considered kinetically indistinguishable.

For the assay reactions the law of mass action can then be written as

$$K = \frac{[P] \cdot [Q]}{[PQ]} = \frac{[P^*] \cdot [Q]}{[P^*Q]},$$

where K is the equilibrium constant of the backward (dissociation) reaction. Its physical dimension is the concentration unit.

Further definitions used are:

- p actual concentration of non-labelled ligand in the incubation mixture
- x ligand concentration in a sample, as established as an assay result
- p* concentration of the labelled ligand in the incubation mixture
- q concentration of binding sites in the incubation mixture
- t_1 duration of the first incubation step (in the absence of labelled ligand)
- t_2 duration of the second incubation step (after addition of labelled ligand)
- k kinetic decay constant of the complexes PQ or P*Q, resp.: $d[PQ]/dt = -k[PQ]$
- B bound activity of the labelled ligand
- S specific activity of the labelled ligand, i.e. activity per concentration unit
- a non-specifically bound fraction of the labelled ligand, which is assumed to be time-independent (10)
- v dilution factor of the non-labelled ligand in the incubation mixture
- ΔB intraassay standard deviation of B
- Δx intraassay standard deviation of x
- ε variation coefficient of a single pipetted volume

Concerning the kinetic equations of the assay reactions, it is very convenient for practical purposes to use k^{-1} as a time unit and K as a concentration unit. An analytical solution of these equations is provided by *Rodbard & Weiss* (8). This solution actually represents a rather complex mathematical expression and has the generalized form $[P^*Q] = f(p, p^*, q, t_1, t_2)$, i.e. bound labelled ligand concentration as a function of reaction parameters. The concentration of totally bound labelled ligand (B/S) can then be calculated from the sum of its specifically ([P*Q]) and non-specifically ($a \cdot (p^* - [P^*Q])$) bound fraction:

$$B/S = (1 - a) \cdot [P^*Q] + ap^* \qquad \text{Eq. (1)}$$

Provided the reaction parameters k and K as well as reactant concentrations are known, bound activity of the labelled ligand as a function of analyte concentration $B = f(vp)$ (standard curve) can be calculated by using Eq. (1).

The intraassay standard deviation Δx of an unknown concentration is connected with the error ΔB of measurement of B via the steepness of the calibration curve (5):

$$\Delta x = v \cdot \Delta B / (\partial B/\partial p) \qquad \text{Eq. (2)}$$

The error ΔB is usually approximated by empirical expressions, $\Delta B = f(B)$ (4, 11–21), and implicates contributions by different errors:

(i) the pipetting error,

(ii) the error of activity measurement and

(iii) errors derived from sample and reagent handling.

Assuming that the work is performed carefully, that modern measuring devices with low noise are used, and that tracer materials with high specific activity are used, the pipetting error becomes dominant in practical terms. It contributes threefold to the total error (pipetting of Q, P*, P), whereas the measuring error contributes only once. Therefore, assuming that the latter is of an order not greater than ε, its influence on the total error will be neglected for the purpose of this work. (Assuming a *Poisson* counting error of $(BT)^{-1/2}$, it can be minimized by applying a sufficiently high counting time, T.). The error ΔB can then be calculated from the absolute pipetting errors $\Delta p = \varepsilon p$, $\Delta p^* = \varepsilon p^*$, and $\Delta q = \varepsilon q$ by quadratic summation of their respective contributions:

$$\Delta B^2 = \varepsilon^2 [(\partial B/\partial p)^2 p^2 + (\partial B/\partial p^*)^2 p^{*2} + (\partial B/\partial q)^2 q^2]. \quad \text{Eq. (3)}$$

Substitution of Eq. (3) into Eq. (2) results in:

$$CV_{rel}(x)^2 = (\varepsilon^{-1} v^{-1} \Delta x/p)^2 = 1 + \frac{(\partial B/\partial p^*)^2 p^{*2} + (\partial B/\partial q)^2 q^2}{(\partial B/\partial p)^2 p^2} \quad \text{Eq. (4)}$$

where $CV_{rel}(x)$ is the variation coefficient of the determination of x expressed in units of ε. Together with Eq. (1), Eq. (4) permits the calculation of the intraassay precision at each point of the standard curve. The mathematical model presented comprises the general case of the two-step (non-equilibrium) assay, including the special case of the one-step assay by setting $t_1 = 0$.

Calculation of the precision index, λ

Intraassay precision may easily be estimated by considering the deviations of experimental points from a suitably fitted standard curve, B = f (vp). Supposing a linear relationship between the appointed standard concentrations, vp_i, and their values, x_i, re-estimated from the fitted standard curve, the precision index of the linear correlation x = a + b (vp) is statistically defined by:

$$\lambda^2 = s_b^2/b^2 = \frac{bv \Sigma (p_i - \bar{p}_i) \Delta x_i + \Sigma \Delta x_i^2}{(n-2) v^2 \Sigma (p_i - \bar{p}_i)^2}, \quad \text{Eq. (5)}$$

where s_b is the standard deviation of the regression coefficient ($b \sim 1$), n is the number of the different standard concentrations with the mean value $\bar{p}_i$, and Δx_i are the deviations of x_i from their appointed values. Averaging over several assay runs, j, the term $\sum_i (p_i - \bar{p}_i) \sum_j \Delta x_{ij}$ will vanish because $\sum_j \Delta x_{ij} = 0$, if the standard curve is properly fitted. Thus the statistically expected value of λ^2 should be

$$\lambda^2 = \frac{\Sigma \Delta x_i}{(n-2) v^2 \Sigma (p_i - \bar{p}_i)^2} \quad \text{Eq. (6)}$$

According to Eq. (6), λ defines an overall precision and can be interpreted as the precision of measurement of x at n different points of the vp-axis which is centred at the point $\bar{p}_i$. It can be pre-calculated by using Eq. (1) and (4).

Calculation of detection limit

As a measure the standard deviation, Δx at p = 0 can be used. (Customarily the two- or threefold value is used, but this is meaningless for the purpose of this study.) From Eq. (4) it follows:

$$\Delta x^2 (p = 0) = \varepsilon^2 v^2 \frac{(\partial B/\partial p^*)^2 p^{*2} + (\partial B/\partial q)^2 q^2}{(\partial B/\partial p)^2} \Bigg|_{p=0} \quad \text{Eq. (7)}$$

Mathematical optimization of assay conditions

In solving the optimization task it is impracticable to simultaneously minimize the complete precision profile, i. e. the standard deviations or variation coefficients in dependence on analyte concentration. Therefore, a target concentration, P, is chosen which is situated in the midrange of the concentrations to be assayed, the standard deviation of which is to be minimized. The optimization problem in hand is multivariate, because four parameters (q, p*, t_1, t_2) have to be varied. Certain considerations may, however, enable its simplification. First, t_2 will be infinite in the case of the one-step method (equilibrium), and t_1 will be infinite in the case of the two-step method (equilibrium reached after completing the first incubation step). Secondly, to ensure an effective competition between P* and P, the concentration p* should be of the order of P or less. The remaining variant q is then the main determinant of achieving an optimal precision profile (9). As a criterion for determination of the optimum q value, minimization of Δx at p = P is taken. The actual optimal values of p*, t_1, t_2 will now depend on q. They should properly be defined by attaining the minimum detection limit at the q value selected. This represents a more preferable criterion for optimizing these parameters than minimization of Δx at p = P, because a good precision profile should provide an agreeable Δx not only at p = P but also at p = 0. In this way, the multivariate optimization problem can be solved by a stepwise quasi-univariate strategy. This is especially important with regard to an experimental optimization strategy which, in principle, can be performed in the same way.

An iterative procedure is recommended, starting with a one-step incubation protocol during which a very low (tracer) p* value and a sufficiently high value of t_2 are employed, while q is being varied. With the established optimal q value, p* can now be adjusted to its ultimate value. The optimization cycle can then be repeated. Changing to the two-step method, a sufficiently high value of t_1 should be used and optimal parameters (q, p*) of the one-step method may serve as starting values for further optimization. Beginning with the determination of an optimum value of t_2, the new values of q and p* can be obtained as in the one-step method. Because of their dependence on q, it is not obligatory to explicitly evaluate p* and t_2 as given in their conventional physical units. They can rather be handled as implicit optimization parameters, knowledge of which is only important in the context of the mathematical procedure. Thus, mathematically as well as experimentally, optimization centres on the evaluation of q values. In particular, it is unnecessary to experimentally determine kinetic dissociation constants, k, unless dependency on time of any assay parameters is considered.

Radioimmunoassay systems

Six radioimmunoassay systems have been developed for the determination of:

(1) human C-peptide (22)
(2) glucagon (23)
(3) rat insulin (24)
(4) human growth hormone (25)
(5) free insulin (26)
(6) human insulin (27)

Standard-curve fitting

A model function $vp = a_{-1}y^{-1} + a_0 + a_1y$ (28) was used, where vp are standard concentrations, y is the response variable of the assay minus non-specific binding, and a_i are constants. This function is derived from the mathematical model described above, but in a strictly simplified form. The coefficients a_i were evaluated by a least-square method taking into account the secondary conditions $a_{-1} > 0$ and $a_1 < 0$, which follow from theoretical reasoning.

It must be pointed out that at higher values of vp (where y becomes near zero), statistical deviations of y will gain a relatively high weight because of the inverse form of our model function. This reflects the high degree of uncertainty of assay results in this region. The range of measurement should therefore not be extended to more than 2P. Keeping that in mind, a theoretically based three-parametric model function is superior to any empirical regression function using a high number of parameters. Such regression functions tend to follow experimental fluctuations and, in our experience, statistical quality parameters often fail to meet their theoretically expected values.

Determination of equilibrium binding parameters, K and q

Competition curves utilizing trace (but known) concentration of labelled antigen and a wide range of concentrations of non-labelled antigen were established at an appropriate antibody dilution. K values as well as antibody binding site concentrations, q, were derived from the left-hand (steeply declining) part of the *Scatchard* plot, where the working range of the antibody is normally situated.

When determined in this way, especially q values may be affected by uncertainty. Therefore we alternatively calculated q values from the parameters of the fitted standard curve (28). The resulting expressions are

$$q = a_{-1} / y_T \qquad \text{Eq. (8)}$$

for one-step assays and

$$q = \sqrt{1/4\, a_0^2 - a_1\, a_{-1}} - 1/2\, a_0 \qquad \text{Eq. (9)}$$

for two-step assays, where y_T is the total tracer activity employed in the assay.

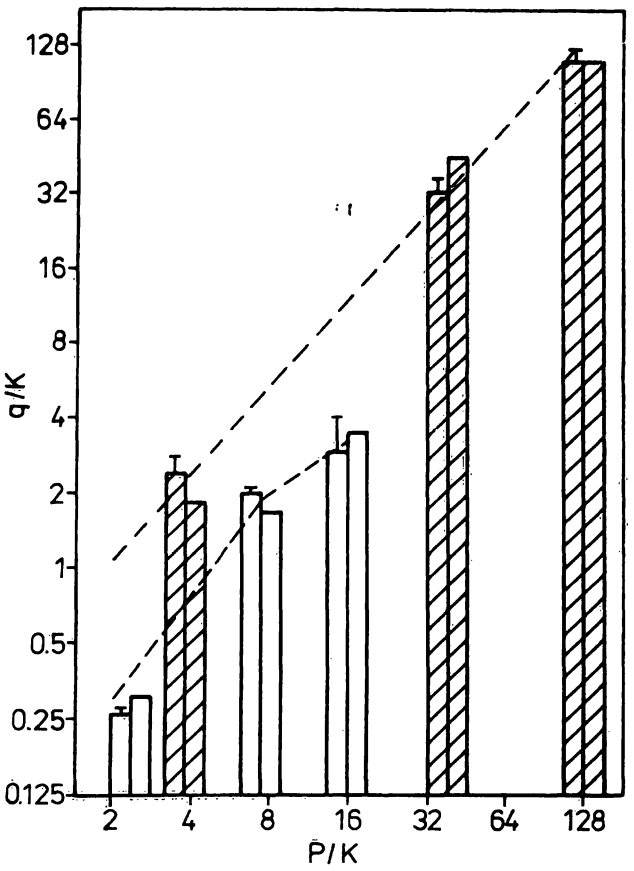

Fig. 1. Optimal antibody binding site concentrations, q, in dependence on target antigen concentrations, P, (both referred to the pertinent equilibrium constants, K) for six RIA systems (from left to right: RIA for human C-peptide, glucagon, rat insulin, human growth hormone, free insulin in human sera, insulin in human sera, see Methods). For each pair of columns, the left one was calculated from parameters of standard curve fitting, whereas the right one was derived from the *Scatchard* plot of a binding curve for either antiserum. Broken lines indicate the values obtained by mathematical optimization. Open and hatched bars represent one-step or two-step assays, respectively.

Results

As illustrated in figure 1, there was an excellent agreement between the optimal concentration of antibody binding sites obtained by experiment and, independently, by mathematical modelling of the 6 RIA systems investigated. Experimental q values were very similar, whether they were derived from *Scatchard* plots or from the parameters of the fitted standard curves. Optimal q values depend on target concentrations, P, both P and q being expressed in units of the equilibrium constants, K. Optimal q values of the two-step method, ranging from P/2 to P, were 3 to 4-fold higher than those of the one-step method, which range from P/8 to P/4. As a result of higher reagent concentrations, the calculated response variable of the assay is about four times higher in the case of the two-step method as presented in figure 2. Precision indices, λ, of the 6 RIA systems investigated are shown in figure 3. In order to refer to the precision of a single measurement, they were multiplied by $\sqrt{N(n-2)} = \sqrt{15}$, because each standard curve was established by using $n = 7$ standard concentrations, each being measured in triplicate ($N = 3$). On the whole, experimentally obtained precision indices compare well with those expected by theory. Up to P/K values as low as two, the λ values barely depend on target concentrations, and they range between two- to threefold of the relative pipetting error, ε. The detection limit is nearly proportional to P in the range of P/K values considered (fig. 4). However, when P/K becomes about two, Δx ($p = 0$) already approaches the value of $\sqrt{2}\varepsilon K$, which is the lowest attainable limit predicted by our theory. For a given value of P, detection limits of two-step assays are generally about

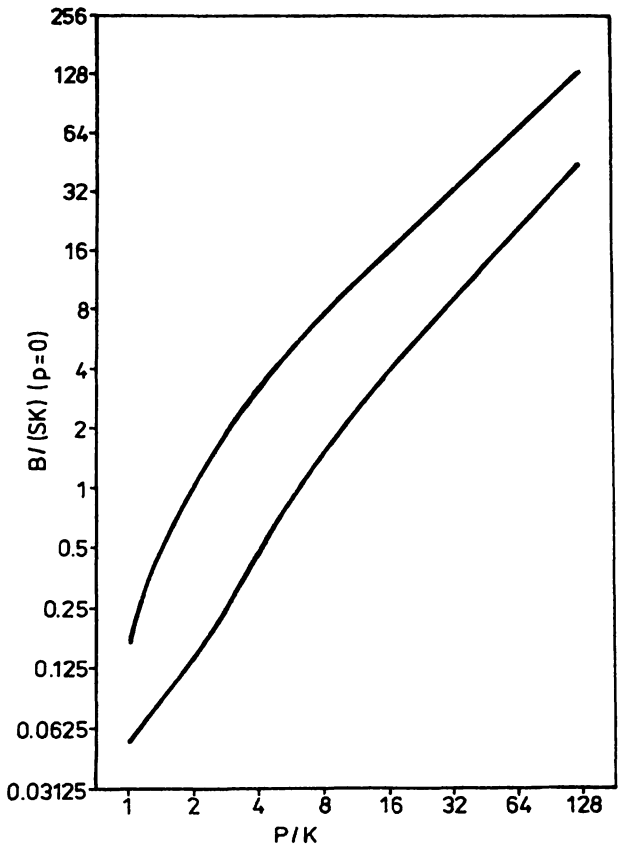

Fig. 2. Theoretically calculated response variable at zero ligand concentration (p = 0) of competitive labelled ligand assays (upper curve: two-step assays, lower curve: one-step assays). Each point of either curve represents bound activity of the labelled ligand, B (p = 0), for an assay targeted to antigen concentration P. Calculations were made according to Eq. (1). B is referred to the product of specific activity, S, and equilibrium constant, K, whereas P is referred to K.

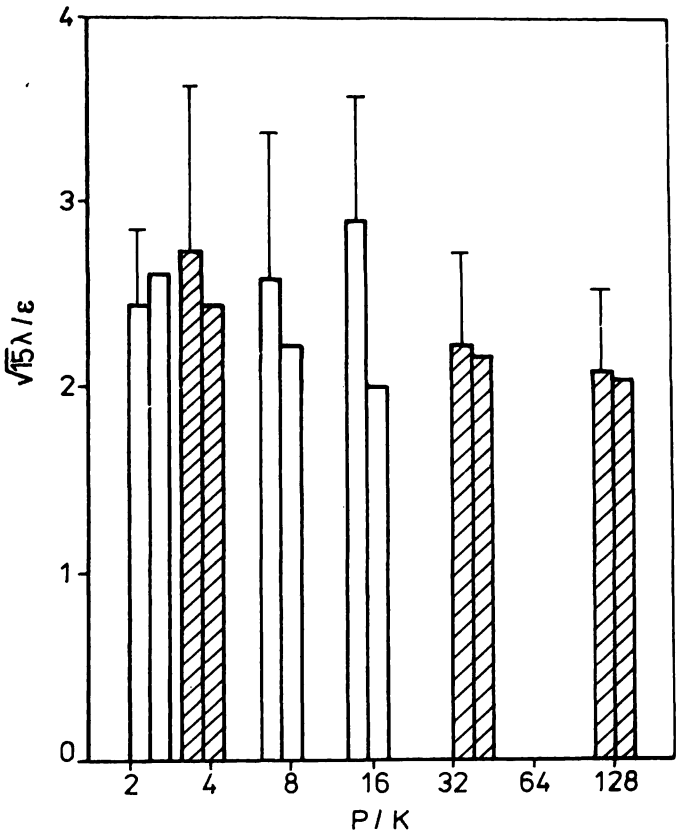

Fig. 3. Precision indices, λ, calculated by Eq. (5) or (6), resp., and given in units of the pipetting error, ε. To make them comparable with the precision of a single measurement, λ values were multiplied by factor $\sqrt{15}$ (see Results). For each pair of columns, the left one represents the mean value of 20 assay runs, whereas the right one was calculated by mathematical modelling.

twice as high (Δx (p = 0) = εP...2εP) as those of one-step assays (Δx (p = 0) = 0.5 εP...εP). Agreement between experimental vs. theoretical values seems to be better for one-step assays.

Figure 5 depicts standard curves and precision profiles of the one-step and two-step variant for an insulin radioimmunoassay. Target concentration was 600 pmol/l (P/K = 92). From 150 up to 600 pmol/l, in practical terms there was no difference in the precision of both forms of the assay. This is the very concentration range where the steepest decline of the standard curve of the two-step assay is situated. However, outside this range the precision of the one-step variant is remarkably better. This corresponds to the lower detection limit of the one-step method as shown in figure 4. On the whole the precision profile of the one-step assay is more favourable than that of the two-step assay.

Concerning assay specificity, we have calculated the cross-reactivity of a ligand B with 100-fold lower affinity to the specific binding site Q than the ligand A (K_B/K_A = 100), which is designated to be both analyte and labelled ligand (fig. 6). In the one-step assay the potency of B as a competitor for binding sites is more than 50 times lower than that of A. However, if the two-step incubation protocol is applied, the reactivity of B is considerably higher. Its potency in comparison with A is now increased and amounts to 1/20 to 1/5 of that of A. Hence, in their ability to discriminate between analyte and cross-reacting substances, one-step assays are superior to two-step assays, when considering the usual case of a cross-reacting ligand of lower affinity than the ligand to be assayed. Table 1 shows the results of the determination of insulin immunoreactivity in samples with rodent insulins when using an RIA system for human insulin. In all cases, immunoreactivity of cross-reacting rodent insulins was significantly lower when determined by the one-step method instead of the two-step method. This demonstrates the higher relative specificity of the assay if the one-step method is used.

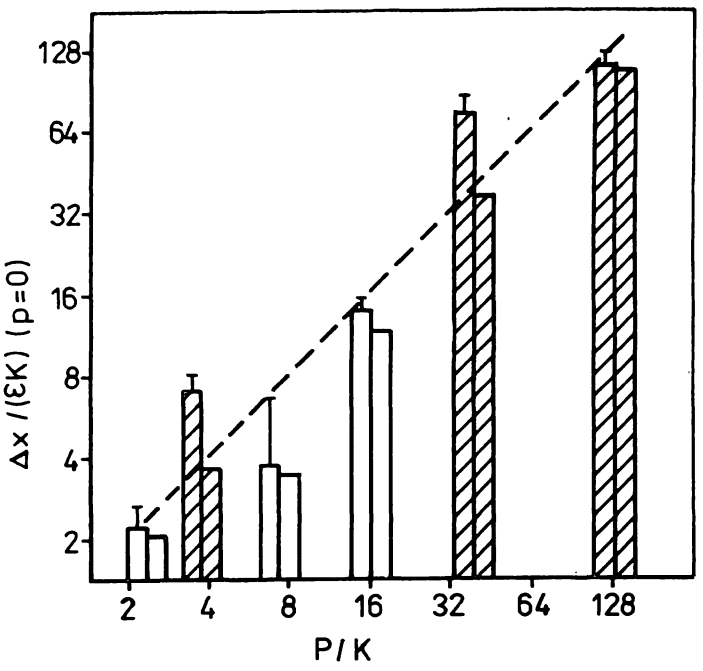

Fig. 4. Standard deviation Δx of the determination of zero ligand concentration (p = 0) for the six RIA systems named in figure 1. Δx is expressed in units of the product of the pipetting error, ε, and the equilibrium constant, K, whereas P is referred to K. For each pair of columns the left one represents the mean value of 20 assay runs, whereas the right one was calculated by Eq. (7). For comparison, the relationship Δx/ε (p = 0) = P is indicated by the broken line.

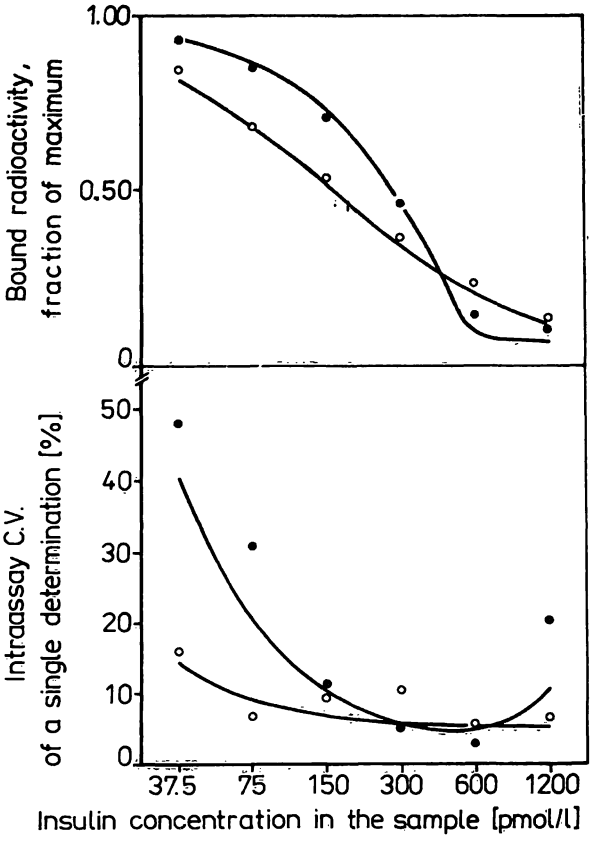

Fig. 5. Experimentally obtained (circles) and theoretically computed standard curves (upper panel) and precision profiles (lower panel) of the one-step (open circles) and two-step variant (closed circles) of an RIA for insulin (non-specific binding: a = 0.9%, pipetting error: ε = 2.8%, P/K = 92).

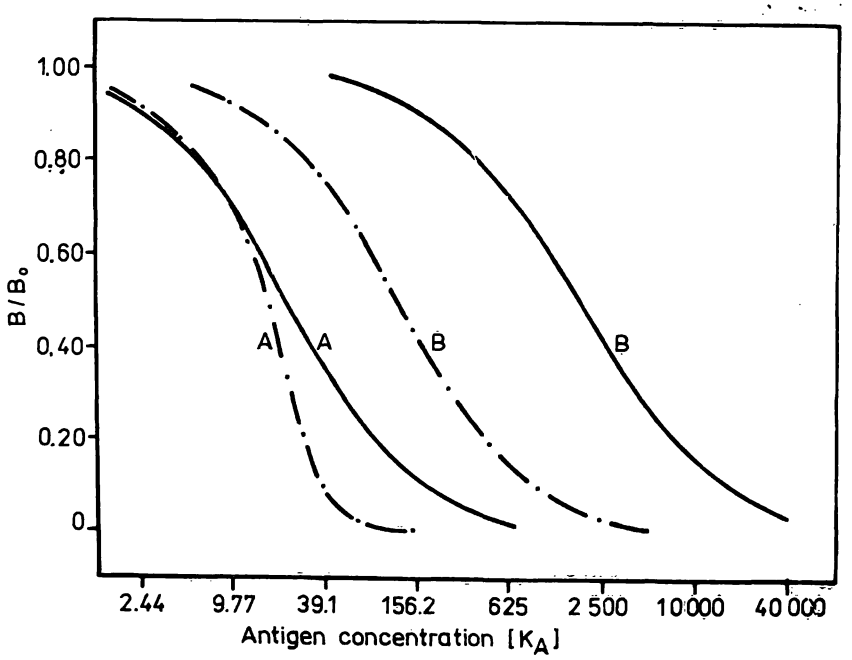

Fig. 6. Competition curves of two ligands, A and B, with different affinities for the specific binding sites, Q, (K_B/K_A = 100) using A* as a tracer ligand. Full lines, one-step assay; dotted lines, two-step assay. Antigen concentrations are expressed in units of the equilibrium constant, K_A, of the reaction between A and Q.

Tab. 1. Determination of insulin concentrations (x ± S.D.) in samples containing insulin from different species, using an RIA for human insulin.

Insulin species	One-step method (pmol/l)	Two-step method (pmol/l)	Significance
Human	502 ± 50	507 ± 84	N.S.
	865 ± 118	891 ± 83	N.S.
Wistar rat	288 ± 38	384 ± 32	$p < 0.001$
	486 ± 46	712 ± 47	$p < 0.001$
Sand rat	222 ± 26	258 ± 38	$p < 0.025$
	446 ± 60	633 ± 30	$p < 0.001$

Discussion

The present mathematical theory of competitive labelled-ligand assays contains several most restrictive assumptions concerning the kinetic behaviour of the reactants (see part "Methods"), which are normally not fulfilled. Neither are the reactants kinetically homogeneous (monoclonal antibodies excepted), and in general, labelled and non-labelled ligand are not kinetically identical, although this would be desirable. Also, non-specific binding may actually be time-dependent inasmuch as separation of bound and free ligand does not always take place instantaneously and can affect the proportion between both forms of the ligand. Nevertheless, it was the goal of our work to prove whether such a simple model can provide results which are acceptable in practice. We have shown our theory to be capable of providing estimates of optimal assay conditions and precision data. After establishing an assay by experiment, it is possible to mathematically re-evaluate the assay conditions. This helps to ensure that they truly result in both optimal reagent (especially antibody) concentration and minimum values of the intraassay precision index and the detection limit. In principle, precalculation of assay conditions requires knowledge of kinetic and equilibrium constants, k and K. Furthermore, to compare theoretically evaluated optimal reagent concentrations with those obtained by experiment, the concentration of both the binding sites of the given antiserum and the antigen content of the labelled ligand solution must be known. As emphasized in the Methods section, however, the main determinant of optimal assay design is the concentration of binding sites, q. While its optimal value is a function of the target concentration, P, the optimal incubation times like the labelled antigen concentration, p*, depend directly on q. As demonstrated in the Methods section, the latter parameters may thus directly be adjusted to the selected antibody concentration not only experimentally but also theoretically. It is therefore neither necessary to experimentally determine the kinetic dissociation constant, k, nor the antigen content of the labelled ligand solution, if comparison of the calculated estimates of t_2 and p* with their experimental values is not intended. An estimate of K, however, has inevitably to be established in order to adjust P and other concentration variables to a K-based concentration scale (*Scatchard* analysis). Similarly, the binding site concentration of the antiserum needs to be determined for the purpose of comparing experimental with theoretical q values.

Precalculated q values compared well with those obtained by experiment (fig. 1). Similar results were obtained when theoretical q values were evaluated, not by the iterative procedure described in the Methods section, but directly by approaching stepwise the minimum of Δx at $p = P$ (with the boundary condition of attaining the best detection limit at the given q value) (data not shown). If the target concentration, P, is higher than the equilibrium constant, K, intraassay precision data can be assessed without knowledge of any kinetic or thermodynamic reaction parametes (figs. 3 and 4), but simply by knowing the relative pipetting error, ε, which may easily be determined. If P becomes of the order of K or lower, intraassay precision indices will, however, rise markedly, and the standard error Δx ($p = 0$), as a measure of the intraassay detection limit, will reach its lowest possible value of $\sqrt{2}\varepsilon K$ predicted by theory. It is thus essential to know at least an approximate value of K in order to assess whether a given intraassay precision index or detection limit may exceed the estimates of figures 3 and 4 for either theoretical or other reasons. It should be pointed out that interassay precision data, which normally are distinctly higher than intraassay data (12), cannot be precalculated by a simple theory, because interassay precision is determined by complex factors such as sample and reagent handling, thermic and time instabilities and others which cannot be assessed without considerable difficulty. On the other hand, interassay precision is a decisive criterion for a useful assay system, and statistical quality control based on interassay data is absolutely necessary.

The range of concentrations where the assay shows the greatest precision is distinctly narrower for the two-step assay (fig. 5), thus favouring the one-step assay for achievement of a good precision profile. This contradicts the common opinion that the employment of the two-step incubation protocol would result in an improved sensitivity of the assay (29). The latter would only be true if identical concentrations of reagents were applied. In fact the optimal

reagent concentrations of a one-step assay are lower than those of the corresponding two-step assay (fig. 1). Keeping that in mind, a better sensitivity of one-step assays than of two-step assays can be predicted when both are targeted to the same analyte concentration, P. When assay sensitivity is limited not by the magnitude of P or the equilibrium constant, K, but by the specific activity of the tracer, the employment of the two-step assay protocol may really increase the sensitivity of the assay. Under this condition, optimal reagent concentrations are only defined by specific activity in that they must be high enough to ensure a still measurable physical signal. In that case, reagent concentrations are identical for both types of the assay protocol and are further from their "optimum values" when the one-step method is applied, resulting in a lower sensitivity. A relatively low tracer activity will thus favour the employment of the two-step incubation protocol, either because the attainable higher measuring signal is higher (fig. 3), or the sensitivity is better. On the other hand, the higher binding site concentration, which is used in two-step assays, causes a lower specificity compared with one-step assays (fig. 6). The excess of binding sites in relation to bound non-labelled ligand molecules existing over a wide range of concentrations, leads to an insufficient competition between molecules of the analyte and of cross-reacting substances. The latter therefore have a statistically greater chance of binding than in the one-step assay, where the number of binding sites is more restricted. Conversely, because of their normally lower affinity, cross-reacting molecules are handicapped in their binding when the one-step technique is applied, resulting in a higher specificity of that method. Thus, it deserves consideration that the relatively high measuring signal provided by the two-step method is ultimately associated with loss of precision, sensitivity and specificity.

References

1. Yalow, R. S. & Berson, S. A. (1959) Assay of plasma insulin in human subjects by immunological methods. Nature *184*, 1648–1649.
2. Schuurman, H. J. & de Ligny, C. L. (1979) Physical models of radioimmunoassay applied to the calculation of the detection limit. Anal. Chem. *51*, 2–7.
3. Jackson, T. M. & Ekins, R. P. (1986) Theoretical limitations on immunoassay sensitivity. Current practice and potential advantages of fluorescence Eu^{3+} chelates as non-radioisotopic tracers. J. Immunol. Meth. *87*, 13–20.
4. Ekins, R. P. & Newman, B. (1970) Theoretical aspects of saturation analysis. Acta Endocrinol. *64*, Suppl. 147, 11–36.
5. Ekins, R. P., Newman, G. B., Piyasena, R., Banks, P. & Slater, J. D. H. (1972) The radioimmunoassay of aldosteron in serum and urine: theoretical and practical aspects. J. Steroid Biochem. *3*, 289–304.
6. Wilson, D., Sarfaty, G., Clarris, B., Douglas, M. & Crawshaw, K. (1971) The prediction of standard curves and errors for the assay of estradiol by competitive protein binding. Steroids *18*, 77–90.
7. Rodbard, D. & Weiss, G. H. (1973) Mathematical theory of immunoradiometric (labelled antibody) assays. Anal. Biochem. *52*, 10–44.
8. Rodbard, D., Munson, P. & de Lean, A. (1978) Improved curve fitting, parallelism testing, characterization of sensitivity and specificity, validation and optimization of radioligand assays. In: *Radioimmunoassay and Related Procedures in Medicine 1977* (IAEA), pp. 469–504, Vienna.
9. Yanashigita, M. & Rodbard, D. (1978) Computerized optimization of radioimmunoassays for hCG and estradiol: an experimental evaluation. Anal. Biochem. *88*, 1–19.
10. Rodbard, D. & Catt, K. J. (1972) Mathematical theory of radioligand assays: the kinetics of separation of bound from free. J. Steroid Biochem. *3*, 255–273.
11. Rodbard, D. & Lewald, J. E. (1980) Computer analysis of radioligand assay and radioimmunoassay data. Acta Endocrinol. *64*, Suppl. 147, 79–103.
12. Rodbard, D. (1974) Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. Clin. Chem. *20*, 1255–1270.
13. Rodbard, D., Lenox, R. H., Wray, H. L. & Ramseth, D. (1976) Statistical characterization of the random errors in the radioimmunoassay dose-response variable. Clin. Chem. *22*, 350–358.
14. Rodbard, D. (1978) Statistical estimation of the minimal detectable concentration ("sensitivity") for radioligand assays. Anal. Biochem. *90*, 1–12.
15. Rolleri, E., Novario, P. G. & Pagliano, B. (1973) Automatic treatment of radioimmunoassay data. J. Nucl. Biol. Med. *17*, 128–141.
16. Keilacker, H., Ziegler, M., Albrecht, G. & Roth, I. (1976) Kinetische Optimierung von Radioimmunoassays unter Verwendung experimenteller Reaktionsparameter. Radiobiol. Radiother. *16*, 125–131.
17. De Lean, A., Munson, P. J. & Rodbard, D. (1978) Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Amer. J. Physiol. *235*, E97–E102.
18. Finney, D. J. (1979) The computation of results from radioimmunoassay. Meth. Inform. Med. *18*, 164–171.
19. Huet, S. (1984) DOSSTAT: a conversational program for statistical analysis of radioimmunoassays. Comp. Progr. Biomed. *18*, 241–246.
20. Kraupp, M., März, R., Legenstein, E., Knerer, B. & Szekeres, T. (1986) Evaluation of radioimmunoassays: comparison of dose interpolation calculations by four parameter logistic and spline functions. J. Clin. Chem. Clin. Biochem. *24*, 1023–1028.
21. Gettys, T. W., Burrows, B. M. & Henricks, D. M. (1986) Variance weighting functions in radioimmunoassay calibration. Amer. J. Physiol. *251*, E357–E361.
22. Rjasanowski, I., Keilacker, H., Besch, W., Richter, K. V., Heinke, P. & Michaelis, D. (1987) Follow-up study of HLA-typed newly diagnosed juvenile Type 1 diabetics over five years. Exp. Clin. Endocrinol. 89, 216–224.

23. Freyse, E. J., Fischer, U., Albrecht, G., Marx, S. & Keilacker, H. (1987) The effect of prehepatic insulin administration on alanine flux rates in diabetic dogs. Diabetologia *30*, 402–408.
24. Gottschling, H. D., Ziegler, M., Wilke, W. & Michael, R. (1974) Radioimmunoassay von Plasmainsulin. Methodenkritische Untersuchungen. Radiobiol. Radiother. *15*, 91–97
25. Woltanski, K. P., Keilacker, H., Ziegler, M. & Kohnert, K. D. (1986) Radioimmunoassay für menschliches Wachstumshormon (HGH). Radiobiol. Radiother. *27*, 215–223.
26. Keilacker, H., Besch, W., Woltanski, K. P., Diaz-Alonso, J. M., Kohnert, K. D. & Ziegler, M. (1987) Measurement of insulin in human sera using a new RIA kit. 2. Determination of free and total insulin – correlations to insulin antibody levels. Exp. Clin. Endocrinol. *90*, 271–277.
27. Besch, W., Woltanski, K. P., Keilacker, H., Diaz-Alonso, J. M., Schulz, B., Amendt, P., Kohnert, K. D. & Ziegler, M. (1987) Measurement of insulin in human sera using a new RIA kit. 1. Insulin determination in the absence of insulin antibodies – conventional assay and micro modification. Exp. Clin. Endocrinol. *90*, 264–279.
28. Keilacker, H., Becker, G., Ziegler, M. & Gottschling, H. D. (1980) Radioligand assays – methods and applications. 4. Uniform regression of hyperbolic and linear radioimmunoassay calibration curves. J. Biochem. Biophys. Meth. *3*, 228–231.
29. Rodbard, D., Ruder, H. J., Vaitukaitis, J. & Jacobs, H. S. (1971) Mathematical analysis of kinetics of radioligand assays. Improved sensitivity by delayed addition of labelled ligand. J. Clin. Endocrinol. *33*, 343–355.

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