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Extension of the four-parameter logistic model for ELISA to multianalyte analysis

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Abstract

The standard implementation of enzyme-linked immunosorbent assay (ELISA) for single analytes can lead to false conclusions if cross reacting compounds are present in the sample. This paper discusses the extension of the usual four-parameter logistic model for ELISA to the case of multiple cross-reacting analytes. The use of the extended model in multianalyte analysis (MELISA) is illustrated and compared with a more simplistic approach. Data on the analysis of a binary mixture of s-triazines suggests the superiority of the proposed model. This model is also suitable for other forms of immunoassay that use the four-parameter logistic curve.

Keywords: Immunoassay; Logistic model; Cross-reactivity

1. Introduction

Enzyme-linked immunosorbent assays are a rapid and sensitive method for quantitating clinical or environmental analytes in trace amounts (Hall et al., 1990; Hammock et al., 1987; Ishikawa et al., 1993; Ishikawa and Kohno,1989; Jung et al., 1989, Vanderlaan et al., 1988; Van Emon et al., 1989). Typically, the dose-response curve obtained is sigmoidal in shape. The four-parameter log-logistic model:

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^{B}} + D \qquad (1)$$

where y is the ELISA response (optical density), x the analyte concentration, A and D the responses at zero and infinite dose, C the IC_{50} (the concentration giving 50% inhibition) and B a slope parameter, has been shown to be a useful

Abbreviations: ELISA, enzyme-linked immunosorbent assay; MELISA, multianalyte ELISA; HRP, horseradish peroxide-labeled; TMB, tetramethylbenzidine; NHS, *N*-hydroxy succinimide; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; MSE, mean square error.

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and flexible tool in assaying the concentration of a single analyte for a variety of ELISA formats (Finney 1978; De Lean et al., 1978; Rodbard 1981). However, it is often observed that the antibodies bind to a number of structurally similar compounds rather than being monospecific for only the analyte of interest, this phenomenon being known as cross reactivity. Thus the standard implementation of ELISA for single analytes can lead to false conclusions if such cross reacting compounds are present in the sample. This problem can be avoided by using an analysis specifically designed to detect and quantitate mixtures of similar compounds.

Extension of the single analyte procedure to the analysis of mixtures has been previously discussed by Rocke (1992) in a theoretical context, Muldoon et al. (1993) for the analysis of mixtures of *s*-triazines in pesticide waste and rinsate, and Cheung et al. (1993) using a complex clustering method. The method proposed by Rocke involved assaying the mixture using a number of different antibodies which have different patterns of affinities towards the analytes. The overall effective concentration for each assay could then be modelled as a linear combination of the concentrations of each analyte using estimated cross-reactivities, so that when the overall response to each antibody is known the unknown concentrations are found by solving a system of linear equations:

$$\begin{aligned} &\text{Response } 1 = R_{11}x_1 + R_{12}x_2 + \dots + R_{1n}x_n \\ &\text{Response } 2 = R_{21}x_1 + R_{22}x_2 + \dots + R_{2n}x_n \\ &\text{etc.} \end{aligned} \tag{2}$$

where x_j is the concentration of analyte j and R_{ij} the cross-reactivity coefficient to antibody i for analyte j.

The problem with this simplistic linear method is that it does not take account of the possibly different slopes of the standard curves. We consider an adaptation of the single-analyte log-logistic curve to incorporate a number of different analytes with different IC_{50}s and possibly different slopes. An empirical comparison is made between the two approaches using analysis of *s*-triazine mixtures.

2. Calculation of 'equivalent concentration'

Suppose we have concentrations x_1 of analyte 1 and x_2 of analyte 2, and that calibration curves of response to a given antibody have been estimated for each analyte using the log-logistic model, i.e., estimates of the parameters A_1, B_1, C_1, D_1 and A_2, B_2, C_2, D_2 are available. If the calibrations are done on the same plate, then it is reasonable to assume $A_1 = A_2$ and $D_1 = D_2$; in fact this condition can be incorporated explicitly into the estimation.

If we wish to estimate the ELISA response y for the mixture we can use the curve for analyte 1, replacing x_2 by the concentration of analyte 1 required to give the same response: the 'equivalent concentration' x_2^*. The expected response is then:

$$y = \frac{A - D}{1 + \left(\frac{x_1 + x_2^*}{C_1} \right)^{B_1}} + D \tag{3}$$

The linear approach suggested by Rocke and implemented by Muldoon et al. is equivalent to taking $x_2^* = (C_1/C_2)x_2$ so that the predicted response from the mixture is:

$$y = \frac{A - D}{1 + \left(\frac{x_1}{C_1} + \frac{x_2}{C_2} \right)^{B_1}} + D \tag{4}$$

This is clearly an unsymmetric arrangement since it uses the slope parameter B_1 from analyte 1, the choice of which is in a sense arbitrary. In particular, if $x_1 = 0$ we would be using the slope from analyte 1 to quantitate a concentration of analyte 2. This method then would be expected to produce bias in the estimated concentrations, particularly for the second analyte.

Consideration of the two standard curves (see Fig. 1) suggests that if x_2 of analyte 2 produces a response y_2, the concentration x_2^* of analyte 1 required to give the same response is given by solving:

$$y = \frac{A - D}{1 + \left(\frac{x_2^*}{C_1} \right)^{B_1}} + D \frac{A - D}{1 + \left(\frac{x_2}{C_2} \right)^{B_2}} + D \tag{5}$$

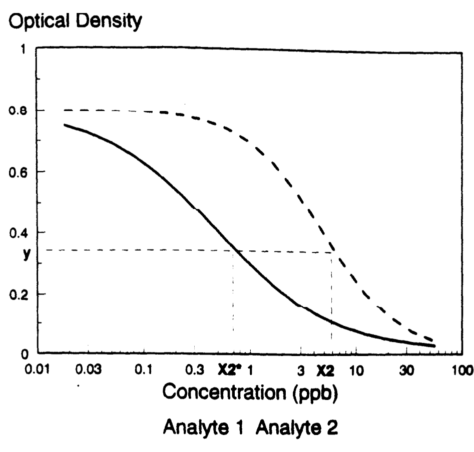

Fig. 1. Calculation of equivalent concentration. x_2^* is the concentration of analyte 1 which gives the same response as a given concentration x_2 of analyte 2. The solid curve is the calibration curve for analyte 1 and the dashed curve is the calibration curve for analyte 2.

i.e.,

$$x_2^* = C_1\left(\frac{x_2}{C_2}\right)^{B_2/B_1} \tag{6}$$

3. The extended log-logistic model

If this equivalent concentration of analyte 1 is now used in the first standard curve we get as the expected response:

$$y = \frac{A-D}{1+\left(\frac{x_1}{C_1}+\left(\frac{x_2}{C_2}\right)^{B_2/B_1}\right)^{B_1}}+D \tag{7}$$

If only analyte 2 were present this reduces to the standard curve for analyte 2. The form of the equation still depends on which analyte is chosen as analyte 1; however, if we choose for our reference curve a third (hypothetical) analyte with slope parameter B^* we get:

$$y = \frac{A-D}{1+\left(\left(\frac{x_1}{C_1}\right)^{B_1/B^*}+\left(\frac{x_2}{C_2}\right)^{B_2/B^*}\right)^{B_*}}+D \tag{8}$$

which satisfies the reasonable condition that it does not depend on arbitrarily choosing one analyte as the standard, as well as reducing to the correct single-analyte standard curve if only one analyte is actually present.

We suggest taking B^* as the geometric mean of the slope parameters of the individual analytes so that the hypothetical reference curve has an 'average' slope.

The solution of an array of equations (as in equation 2) for unknown concentrations $x_1, \ldots, x_n$ is now non-linear, but can be accomplished fairly easily using a standard non-linear equation-solver or minimization routine. The question to be addressed is whether incorporation of different slopes into the model actually does lead to significantly better estimates.

4. An empirical comparison

4.1. MELISA format

For the binary mixture analysis immunoassay we utilized two different monoclonal anti-triazine herbicide antibodies. The two analytes chosen were atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) and terbutryn (2-*tert*-butylamino-4-chloro-6-ethylamino-1,3,5-triazine), two triazines with different substitution patterns. Mixture analysis requires antibodies that have sufficiently different response patterns to the different analytes. This was achieved using Mab K1F4, which was more specific for terbutryn than for atrazine (Giersch and Hock, 1990), and Mab AM7B2.1 which was more specific for atrazine (Karu et al., 1991).

The competitive assay was based on the coating hapten format which requires simultaneous incubation of the analyte and the specific antibody on microtiter plates coated with an analyte analogon (hapten) coupled to a carrier protein (coating hapten). In the second step a secondary, horseradish peroxide-labeled (HRP) anti-mouse IgG was introduced. The last step was substrate conversion of tetramethylbenzidine (TMB) into a colored dye and stopping the reaction with acid.

Table 1
Experimental design

Mixture	Atrazine (ppb)	Terbutryn (ppb)
1	0.0	0.0
2	0.0	0.1
3	0.0	0.5
4	0.0	3.0
5	0.1	0.0
6	0.1	0.1
7	0.1	0.5
8	0.1	3.0
9	0.5	0.0
10	0.5	0.1
11	0.5	0.5
12	0.5	3.0
13	3.0	0.0
14	3.0	0.1
15	3.0	0.5
16	3.0	3.0

The 16 mixtures used as 'unknowns' consists of all combinations of 0, 0.1, 0.5, and 3.0 parts per billion each of atrazine and terbutryn.

The coating hapten was a triazine derivative conjugated to ovalbumin, prepared in a standard *N*-hydroxy succinimide (NHS) ester method (Tijssen, 1985). A hapten suitable for both antibodies was chosen after the cross-reactivity for each antibody towards a panel of different triazine derivatives was evaluated. The criteria for suitability included a low curve midpoint (IC_{50}) for both analytes (in the low ppb range) and the coverage of compatible dynamic ranges for both antibodies.

To perform the immunoassay, standard curves of both analytes were run on two separate microtiter plates together with the same set of unknowns: one plate using K1F4, the other AM7B2.1. The 'unknown' mixtures used are shown in Table 1.

4.2. Assay protocol

Microtiter plate wells were coated overnight at room temperature by incubating 180 μl of coating hapten solution in phosphate buffered saline (PBS) pH 7.4. For the subsequent blocking step 180 μl of a 0.5% ovalbumin-PBS solution were incubated for 1 h. The plates were rinsed four times with washing buffer (0.1 PBS solution containing 0.05% of the detergent Tween 20) and were ready to use. Triazine standards were prepared by diluting a *N,N*-dimethylformamide (DMF) stock solution of 1 mg/ml atrazine and terbutryn respectively in PBS buffer. Standards and mixtures were prepared freshly prior to each assay. 100 μl of standards and unknowns were pipetted in triplicates. KIF4 was diluted 1/8000 and AM7B2.1 1/400 in PBS. 50 μl of the antibody solution was added to the triazine solutions and incubated for 1 h. The plates were rinsed four times with washing buffer and subsequently the anti-mouse IgG-HRP conjugate was introduced. The HRP-anti-mouse IgG conjugate was diluted 1/18000 in PBST buffer (PBS containing 0.05% Tween 20), 100 μl of which was applied per well. After 1 h the plates were washed four times and 100 μl of a substrate solution were applied. The substrate solution was prepared by adding 2.4 mg TMB dissolved in 400 μl dimethyl sulfoxide (DMSO) and 100 μl 1% H_2O_2 to 25 ml sodium acetate buffer pH 5.5. After 15–30 min the enzymatic reaction was stopped by adding 50 μl 4 N H_2SO_4. Photometric measurement was performed in a UV Max 96 well plate reader (Molecular Devices) at 450 nm with a 650 nm background correction.

4.3. Results

For each plate two log-logistic standard curves were fitted using a non-linear maximum likelihood routine (Bunch, Gay and Welsch, 1993). A constant coefficient of variation model was assumed (see O'Connell et al., 1992). The horizontal asymptotes to the logistic curves (A and D) were constrained to be the same for each curve

Table 2
Estimated parameters for standards curves

	A	B_a	B_t	C_a	C_t	D
Plate 1	0.750	0.868	1.116	1.215	0.457	0.049
Plate 2	0.697	1.108	1.010	0.255	1.186	0.049

A and D were common to the two standards on each plate, B_a, and C_a were for atrazine standards, B_t and C_t were for terbutryn standards. Plate 1: K1F4 (anti-terbutryn). Plate 2: AM7B2.1 (anti-atrazine).

on the plate. The parameters are shown in Table 2, where B_a and C_a are the slope and IC_{50} for atrazine, B_t and C_t those for terbutryn. The 'unknown' concentrations were estimated using three different methods of analysis:
Method m using the extended log-logistic model (equation 7) and a non-linear minimization routine;
Method a using the simplistic linear model with atrazine as the chosen standard;
Method t as for method a but using terbutryn as the standard.

Methods a and t occasionally lead to negative estimated concentrations when the actual concentrations are small. When this occurs it seems reasonable to take the estimate as zero, and this has been done for the comparisons here. The results are illustrated in the graphs in Figs. 2 and 3, which show the amount found plotted against the amount added for each analyte and each method. The added lines represent 'perfect' estimation ±20% error.

As expected all methods sometimes failed to detect concentrations of 0.1 ppb, since this was known to be the approximate limit of detection for each individual analyte in a single-analyte ELISA. However concentrations of 0.5 ppb were detected and usually estimated quite well, except for mixture 12 where the actual atrazine concentration of 0.5 was estimated as 0.13 by methods m and t, and missed altogether by method a.

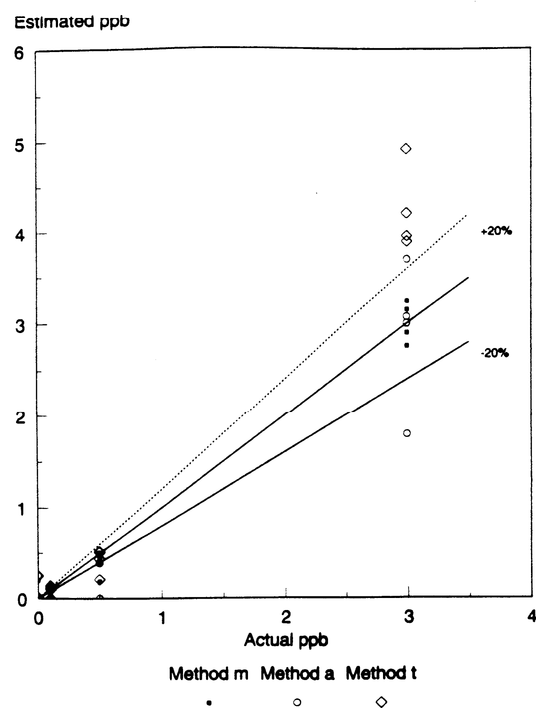

Fig. 2. Estimation of atrazine in 16 mixtures by three methods. method m is the newly proposed 'MELISA' method, methods a and t assume constant cross reactivity. Method a standardizes using the slope of the atrazine calibration curve, and method t standardizes using the slope of the terbutryn calibration curve.

4.4. Comparisons

If we first compare the estimation of atrazine concentrations for the three methods, we can see (Fig. 2) that for higher concentrations (3 ppb) method m gives acceptable results, the method a values are more dispersed and method t gives a clear upward bias (i.e., over-estimation).

For estimating terbutryn, method t seems slightly better than method m, but now method a produces considerable upward bias.

Regression of amount found on amount added (Table 3) again suggests that method m performs well for both analytes, whereas method a is biased for estimating terbutryn and method t for estimating atrazine.

As a final means of comparison we consider the mean squared error (MSE), which gives a measure of the size of the errors in estimating each concentration. It seems reasonable to suppose that the absolute errors increase in proportion to the true concentration, so here the MSE is compared separately at each level (Table 4). This shows that method m was clearly superior for atrazine, and that method a was disastrous for terbutryn.

5. Discussion

It has been shown that a mixture of two analytes that are cross-reactive for two different immunoassays can be analysed using the results

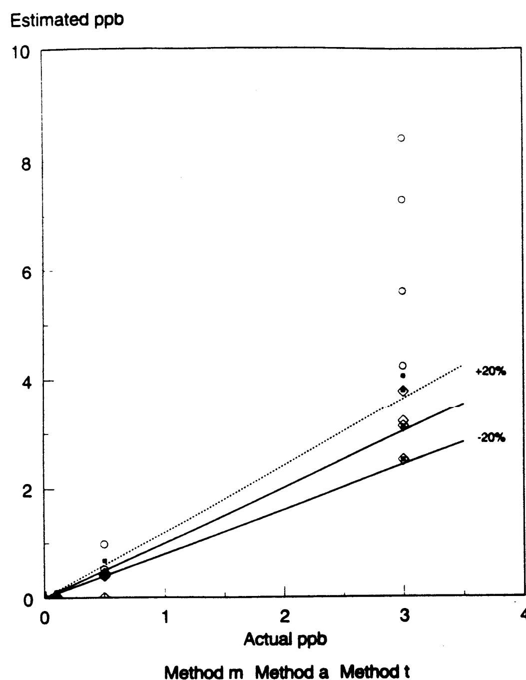

Fig. 3. Estimation of terbutryn in 16 mixtures by three methods. method m is the newly proposed 'MELISA' method, methods a and t assume constant cross-reactivity. Method a standardizes using the slope of the atrazine calibration curve, and method t standardizes using the slope of the terbutryn calibration curve.

from the two assays together with appropriate standard curves; further that this can be performed reasonably accurately even for concentrations as low as 0.5 ppb.

Table 3
Regression of amount recovered on amount added for the 16 unknowns

	Method m	Method a	Method t
Atrazine			
Slope	1.01	0.96	1.43
Intercept	−0.04	−0.02	−0.08
R^2	0.99	0.91	0.98
Terbutryn			
Slope	1.12	2.16	1.06
Intercept	−0.04	−0.22	−0.09
R^2	0.95	0.91	0.96

Method m is the MELISA method. Methods a and t assume constant cross reactivity, with method a standardized using atrazine and method t standardized using terbutryn.

Table 4
Mean square error of the estimated concentrations

	Method m	Method a	Method t
Atrazine			
0.0	0.010	0.016	0.016
0.1	0.008	0.003	0.003
0.5	0.028	0.066	0.022
3.0	0.037	0.490	1.721
Terbutryn			
0.0	0.002	0.000	0.000
0.1	0.008	0.010	0.009
0.5	0.014	0.063	0.067
3.0	0.464	13.550	0.210

For each concentration of atrazine (respectively terbutryn) in the 16 mixtures, this table gives the mean square difference between the amount added and the amount estimated. Method m is the MELISA method. Methods a and t assume constant cross reactivity, with method a standardized using atrazine and method t standardized using terbutryn.

In solving for the unknown concentrations one can either ignore the different slopes of the standards curves and work with linear 'equivalent concentrations', or adopt a slightly more complex analysis (e.g., the proposed extended log-logistic model) which takes account of the different slopes.

Our results suggest that if the simplistic method is used, this can result in serious bias, particularly for the analyte not chosen as the reference standard. This can occur even when the slopes are not greatly different: the slope parameters in our example ranged from 0.87 to 1.12, yet this is enough to cause the substantial bias noted.

The direction of bias (under- or over-estimation) is not easy to predict because of the way the two estimated concentrations interact in the equations. If one were really interested in only one of the analytes, reasonable results might be obtained by using it as the reference standard, but this is by no means certain; otherwise one would have to choose a particular reference standard at random, and depend on luck. It seems preferable then to adopt a more sophisticated model in which all the information in the separate standards curves is used in a balanced, symmetrical analysis.

The proposed model can be extended easily to more complex mixtures, and can be applied in

any clinical or environmental analysis where cross-reacting analytes have to be distinguished and quantitated with as few assays as possible.

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