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Breast Cancer Estrogen Receptor Assays: Assessment of a Direct Hyperbolic Fitting to Analyse Multipoint Binding Data

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Abstract—*Analysis of breast cancer estrogen receptor multipoint binding assay is performed by fitting experimental data to a hyperbolic model derived from the law of mass action. The calculations performed on a microcomputer are carried out from the total bound and free ligand concentrations. The parameters estimated by hyperbolic fitting, receptor concentration* N *and constant of dissociation* K, *well agree with those obtained by Scatchard's transformation.* N *and* K *derived from hyperbolic analysis are much less susceptible to the influence of experimental errors. The method is more reliable at low receptor concentrations. The main advantage of the hyperbolic fitting is to simplify the technical methodology in clinical laboratory practice; there is no need to determine the non-specific bindings experimentally. Calculations can be easily automated on any laboratory microcomputer. Assays of any kind of receptor could be analysed by the hyperbolic fitting when the physical–chemical equilibrium between receptor, nonsaturable component and ligand can be approximated by a two-component model.*

INTRODUCTION

THE MOST common assay for the measurement of estrogen receptors is the dextran-coated charcoal method (DCC). Data obtained from this multipoint binding assay are usually analysed using a mathematical linear transformation to derive the concentration of specific binding sites N and the dissociation constant K. Linear methods, Scatchard plot [1], Wolf plot [2] or reciprocal plot [3], are available only when performed from the specific receptor binding data. They obviously require the measurement of the non-specific binding components (NSB), substances which bind steroid with a relatively low affinity and high capacity, but without relation to the hormone activity. NSB is subtracted from each experimental data-point to derive the specific binding data used for the linear transformation.

Here we describe a mathematical analysis of DCC binding data that removes the need to measure NSB experimentally. The method is based on a hyperbolic relation between the total bound-ligand/free-ligand ratio and the free-ligand concentration, a relation derived from the law of mass action.

Estimations of estrogen receptors in breast tumor had been performed by this method. The results, N and K, were compared to those calculated by the Scatchard transformation routinely used in our laboratory for the past 5 years.

The influence of both Scatchard transformation and hyperbolic analysis on the accuracy and precision with which the parameters N and K could be estimated was investigated by means of experimental errors simulations. The repercussion of non-specific binding levels was also investigated.

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MATERIALS AND METHODS

Biochemical assay of estrogen receptors

Preparation of tissue cytosol sample. Frozen tumor tissue was mechanically pulverized in an impact grinder at liquid nitrogen temperature (Freezer/Mill, SPEX Industries Inc.), then homogenized at 4°C in 3 vol. of Tris buffer (Tris–HCl 10^{-2} mol/l, EDTA 1.5×10^{-4} mol/l, dithiotreitol 1.2×10^{-3} mol/l, KCl 0.4 mol/l, pH 7.4) using an ultra turrax homogenizer. Homogenate was centrifuged at 105 000 *g*, 4°C, for 1 h (Beckman L8–55, Beckman Instruments Inc.). Supernatant was finally diluted in 6 vol. of the Tris buffer plus glycerol 10%.

Assay of estrogen receptor. All assay steps were carried out at 0°C. Each assay required three sets of seven triplicate tubes. In the first set used to measure the total estrogen binding, 150 µl of cytosol were added to 50 µl of [^{3}H]17β-estradiol (New England Nuclear Chemical Gmbh) giving final assay concentrations ranging from 0.12 to 4.4 × 10^{-9} mmol/l. The non-specific binding was evaluated by the second set: each tube contained the same concentration of tracer plus 200-fold excess of unlabelled 17β-estradiol (Sigma Chemical Co.). Results of these 'non-specific tubes' were used only for the estimation of N and K by Scatchard analysis. The last set was used to determine the charcoal-free hormone extraction capacity: 150 µl of Tris buffer instead of tissue cytosol was added to the 50 µl of labelled 17β-estradiol.

After incubation during 16 h at 0°C, an aliquot of 50 µl was taken from each tube to determine the total amount of ligand present. Then 150 µl of dextran-coated charcoal Tris buffer solution (charcoal 1.25%, dextran 0.65%, gelatin 0.2%) were added in the remaining incubates. The tubes were shaken for 10 min and centrifuged for 10 min at 2000 ***g***. An aliquot of 200 µl was taken from each supernatant and counted in a liquid-scintillation counter (model TRI-CARB 460 C, Packard Instrument Co.).

Hyperbolic analysis of data

The hyperbolic model. The hyperbolic model was derived from the generally admitted two-component model representing the reaction:

$$R + NS + H \rightleftarrows HR + HNS \qquad (1)$$

where R is the receptor, NS the non-specific component, H the ligand, HR and HNS the receptor–ligand and the non-specific component–ligand complexes respectively. Application of the law of mass action to the equilibrium (1) leads to the following relation between the total concentration of bound-ligand B_T and the concentration of free-ligand F:

$$B_T = N{\cdot}F/(K+F) + NS{\cdot}F/(K_{NS}+F) \qquad (2)$$

In the first term, which represents the concentration of ligand bound to the receptor, N and K are the concentration of total receptor and the dissociation constant of the HR complex respectively. The second term corresponds to the concentration of ligand bound to the non-specific component, and NS and K_{NS} are the concentration of non-specific sites and the dissociation constant of the HNS complex.

F can be neglected compared to K_{NS}, since F is always inferior to 5 nmol/l in our experimental conditions, and K_{NS} is about a few umol/l as it is generally admitted.

Equation (2) becomes:

$$B_T = N{\cdot}F/(K+F) + NS{\cdot}F/K_{NS} \qquad (3)$$

The hyperbolic relation between B_T/F and F is finally obtained by dividing expression (3) by F:

$$B_T/F = N/(K+F) + NS/K_{NS} \qquad (4)$$

The fitting method.

The fitting method is derived from the so-called "linearization of a regression by the finished increments method" [4].

At first to simplify the symbolism, we put $y = B_T/F$, $x = F$, $a = NS/K_{NS}$. Equation (4) becomes:

$$y = N/(K+x) + a \qquad (5)$$

If x_0, y_0 is a reference pair which verifies equation (5), the transformation $z = (x-x_0)/(y-y_0)$ leads to the following linear relation between z and x:

$$z = i + px$$

where $i = K/(a-y_0)$ and $p = 1/(a-y_0)$. Thus, taking as x_0, y_0 one of the x,y experimental pairs (i.e. $F,B_T/F$ pairs), the linear regression between z and x gives an estimation of i and p and consequently that of K and a.

N is estimated by the relation:

$$N = ((x+K)\ (y-a))/n$$

where n represent the number of experimental pairs.

Calculations were performed on an IBM PC microcomputer (IBM United Kingdom Inter. Prod. Ltd) and carried out using as x_0,y_0 reference, each possible x,y experimental pair successively. Outliers can be excluded if necessary. The medians of results gave the best estimates of N and K, and a range of their values was also obtained. The statistical adequacy of the fit was verified by Student's t test (comparison to zero of the deviations between experimental binding data and those calculated by the equation (4) with the best estimated parameters). The listing of the program run in BASIC is available on request.

To avoid subjectivity the computer was also used to estimate N and K by Scatchard's method.

Experimental error simulations

The repercussion of experimental errors on the determination of N and K by both Scatchard and hyperbolic methods were investigated in the following way. Using the two-component model, total and non-specific theoretical binding data were computed from given N, K, NS and K_{NS} values of fictitious cytosol, and seven concentrations of ligand ranged from 0.175 to 5 × 10^{-9} mol/l. Simulated experimental binding data were then generated by adding to theoretical data, random uncertainties of known maximal magnitude (chosen to equal observed experimental errors). K and N were estimated from these data by the two methods of

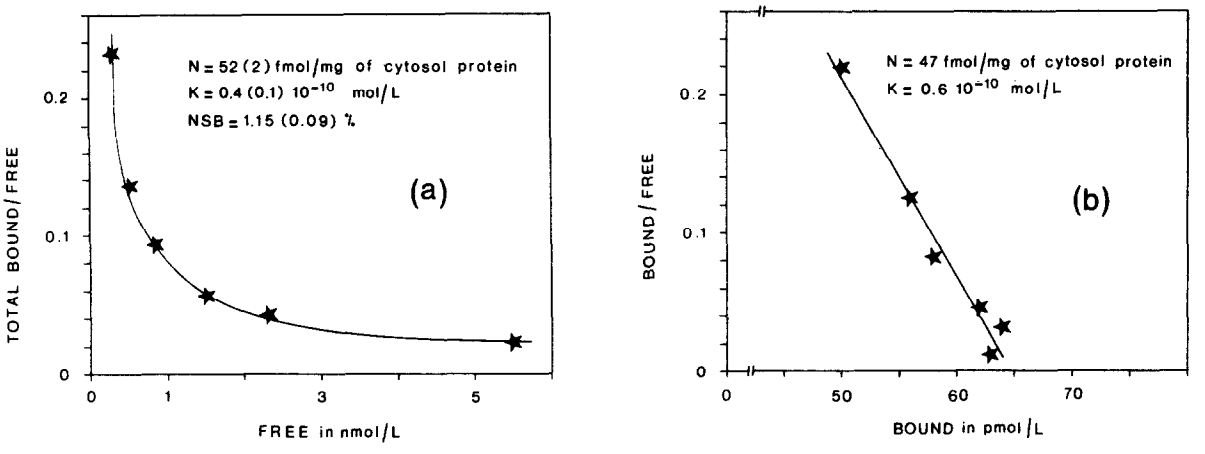

Fig. 1. (*a*) *Typical estimation of receptor parameters derived from the hyperbolic fitting method. Computation leads to total receptor concentration* N, *dissociation constant* K, *non-specific binding level NSB%, and to a range of their values (in parentheses).* (*b*) *Comparative Scatchard plot derived from the specific binding data.*

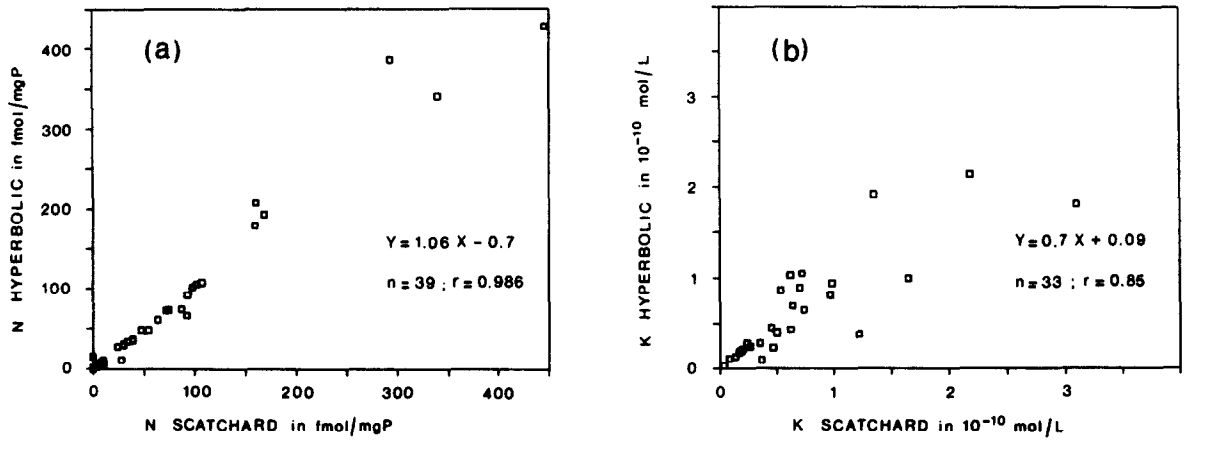

Fig. 2. *Correlation between hyperbolic analysis and Scatchard transformation for* 39 *consecutive estimations of estrogen receptor parameters: concentration* N (*a*), *constant of dissociation* K (*b*).

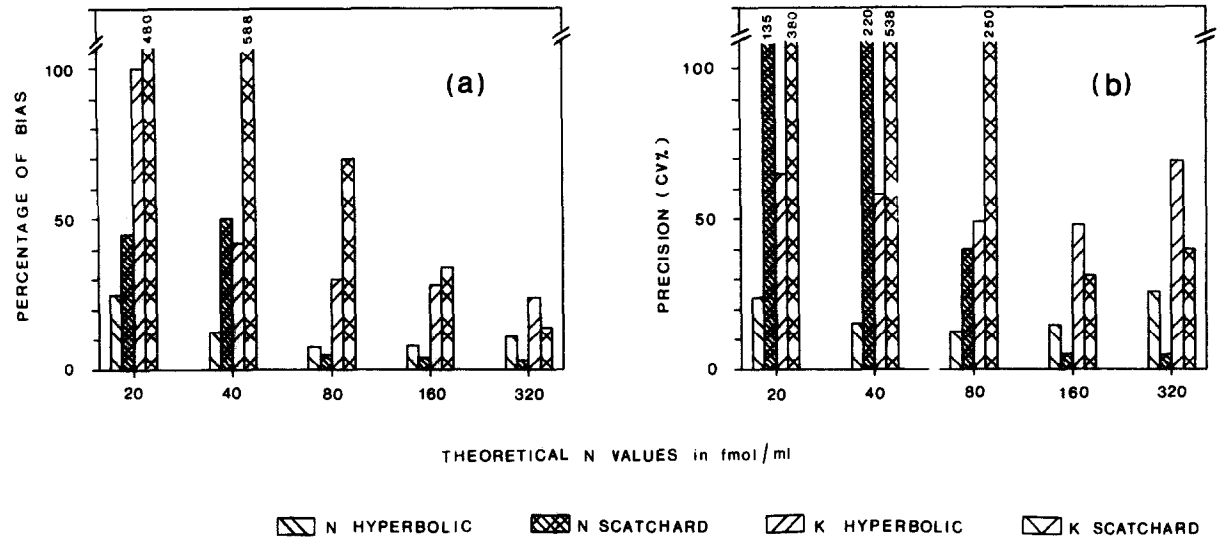

Fig. 3. *Incidence of receptor concentration on bias* (*a*) *and precision* (*b*) *in* N *and* K *estimated from simulated error experiments. Comparison between our hyperbolic method and the Scatchard transformation. Parameters for simulations were:* $K = 0.7 \times 10^{-10}$ *mol/l,* $K_{NS} = 50 \times 10^{-6}$ *mol/l,* $NS = 10^{-6}$ *mol/l* ($NS/K_{NS} = 2\%$), *maximal magnitude for binding data uncertainties calculations* 10%, N *ranged from* 20 *to* 320 *fmol/ml of cytosol.*

analysis and compared to their theoretical values. Up to 100 simulations were run for any data-set. Bias (mean deviation from the theoretical values) and precision (expressed as a coefficient of variation) were calculated for each simulation assay to quantify the repercussion of experimental errors.

RESULTS AND DISCUSSION

An example of N and K estimation by hyperbolic analysis if shown on Fig. 1 (a). In this assay the parameters N and K were 52 fmol/mg of cytosol protein and 0.4×10^{-10} mol/l respectively. For comparison they were 47 fmol/mgP and $0.6\ 10^{-10}$ mol/l when estimated by the Scatchard transformation (Fig. 1(b)).

Hyperbolic and Scatchard correlation

N and K had been estimated in 39 tumors by hyperbolic and Scatchard parallel analysis consecutively. Values derived from the two methods were generally in good agreement (Fig. 2); the coefficient of correlation was $r = 0.98$ for N and $r = 0.82$ for K. When analysed by the hyperbolic fitting method the mean dissociation constant for the binding of 17β-estradiol with the estrogen receptor was 0.70 ± 0.10 (S.E.M.) 10^{-10} mol/l. It was $1.00 \pm 0.24 \times 10^{-10}$ mol/l by Scatchard analysis. Of the 39 results only one discrepancy had been observed: N was equal to 16 fmol/mg of cytosol protein by hyperbolic analysis, while it was found undetectable by Scatchard.

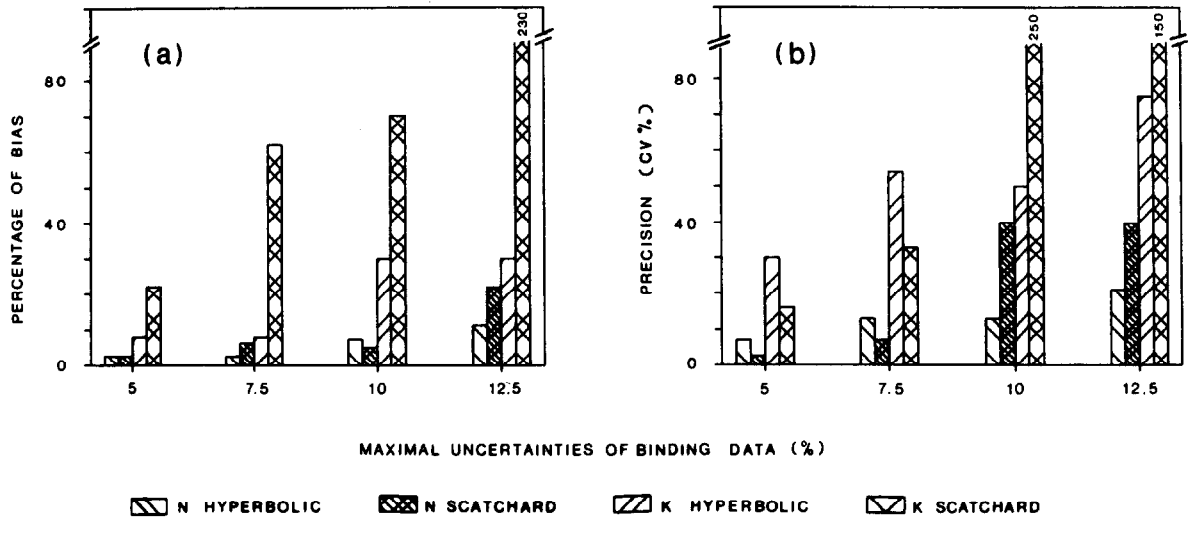

Fig. 4. Comparative change in bias (a) and precision (b) of parameter estimations as the uncertainties were increased insimulated error experiments. Parameters for simulations were: K = 0.7×10^{-10} *mol/l*, $K_{NS} = 50 \times 10^{-6}$ *mol/l*, NS = 10^{-6} *mol/l* (NS/K_{NS} = 2%), N = 80 *fmol/ml of cytosol, maximal magnitude for binding data uncertainties calculations ranged from* 5 *to* 12.5%.

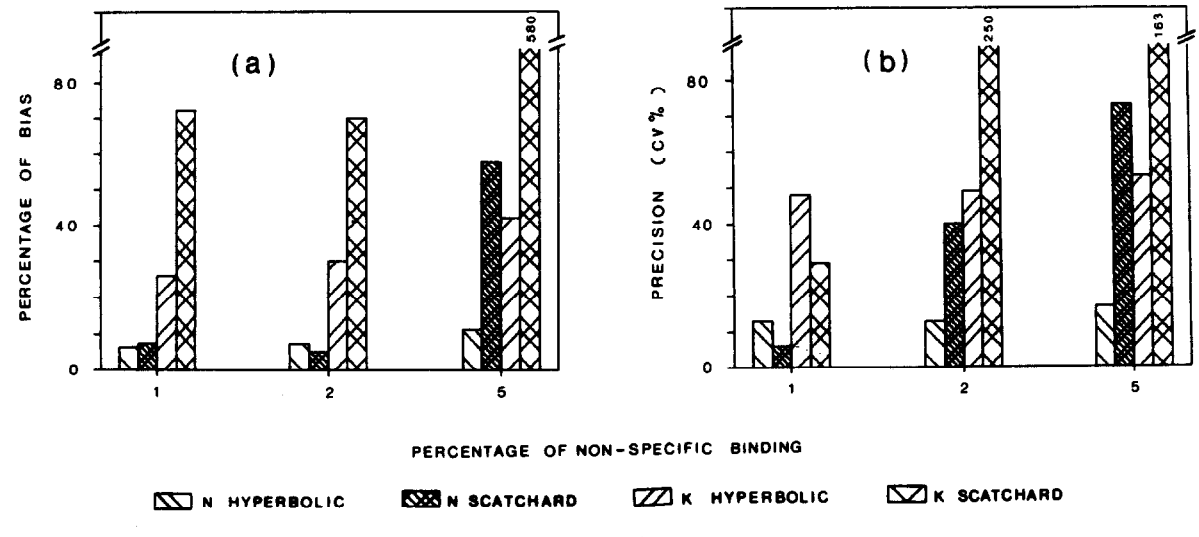

Fig. 5. Comparative incidence of non-specific binding levels on bias (a) and precision (b) of parameter estimated by hyperbolic and Scatchard methods. Parameters for simulations were: K = 0.7×10^{-10} *mol/l*, $K_{NS} = 50 \times 10^{-6}$ *mol/l*, N = 80 *fmol/ml of cytosol, maximal magnitude for binding data uncertainties calculations* 10%. NS *ranged from* 0.5×10^{-6} *to* 2.5×10^{-6} *mol/l* (NS/K_{NS} from 1 to 5%).

Simulated error experiments

C.V. values and deviations from theoretical N and K values were first estimated for values of N ranging from 20 to 320 fmol/ml of cytosol and a maximal magnitude of 10% for binding data random uncertainties calculations. The other parameters used for computations were $K = 0.7\ 10^{-10}$ mol/l, $K_{NS} = 50 \times 10^{-6}$ mol/l, $NS = 10^{-6}$ mol/l ($NS/K_{NS} = 2\%$) (Fig. 3). For all simulation assays, the mean coefficient of variation and the mean bias in the estimation of N were 81% (extreme values 5 and 220%) and 21% (3–45%) using the Scatchard transformation. They were 19% (13–26%) and 13% (7.5–25%) by hyperbolic analysis. Differences in the mean C.V. values and deviations were greater in the estimation of K, being 250% (31–538%) and 237% (14–588%) by Scatchard, only 58% (48–61%) and 45% (24–100%) by the hyperbolic method. Differences in the estimation of N and K between the two methods were generally greater for the low concentrations of receptors.

In order to investigate how the magnitude of binding data uncertainties affected N and K estimations, we simulated experimental errors with different maximal magnitude from 5 to 12.5%. C.V. values and bias increased more rapidly when using the Scatchard transformation (Fig. 4).

The effect of non-specific binding levels was also investigated. C.V. values and deviations derived from the hyperbolic analysis were moderately affected by the increasing of non-specific; in contrast, the Scatchard transformation led to larger variations (Fig. 5).

Further evidence that our hyperbolic method is more robust was obtained by the simulated experimental errors investigations. The parameters N and K estimated by this analysis were much less susceptible to the influence of experimental errors than those estimated by the Scatchard transformation. This is due to the diminution of the uncertainties by eliminating the need to subtract non-specific binding values from each data-point.

In conclusion, estrogen receptors in breast tumor can be advantageously evaluated by our method in routine laboratory practice. The methodology is simplified: only one set of tubes is needed to measure the total binding with increasing concentrations of radioligand. The hyperbolic analysis is more reliable for the determination of low receptor concentrations. The automation of the mathematical hyperbolic analysis can be easily performed on any laboratory microcomputer. Assays of any kind of receptor could be analysed by our hyperbolic fitting method when the physical-chemical equilibrium between receptor, non-saturable component and ligand can be approximated by a two-component model.

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