STATISTICAL TEST OF MODELS AND COMPUTERISED PARAMETER ESTIMATION FOR ALDOSTERONE BINDING IN RAT KIDNEY

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1. Introduction

Quantitative analysis of receptor–ligand interactions is a determinant step for a better understanding of hormonal steroid mechanism of action. Two related problems have to be considered:

1. What type of model is consistent with the experimental data.
2. Once a model has been accepted, what are the corresponding values of the parameters and what are their confidence limits.

The simplest case occurs when the receptor–ligand interaction can be adequately described by a single type of specific binding site which is defined by the relation $B_s = N.U/K_d + U.B$, concentration of steroid bound to the receptor; U concentration of free steroid; K_d, dissociation constant; N, maximum number of receptor sites. In that case, the Scatchard method [1] is applied and consists in a transformation of the data leading to a linear relationship between the new variables: $B/U = f(B)$. Classical linear estimation permits therefore straightforward calculations of unknown parameters: K_d and N.

When multi-site systems interact with one steroid, this method is no longer valid. Many authors, assuming non-interacting models, have proposed either graphical resolution of curvilinear Scatchard plots [2–4] or numerical calculation by least square analysis [5–8]. Some others proposed different types of graphical representation, involving classical mathematical or statistical methods [9–11].

Non-specific binding which can be described by the relation $B_{ns} = \beta.U$ (β, constant) corresponds to a very low affinity and unsaturable binding, frequently implicated in protein–steroid interactions. It is usually estimated by experimental procedure (incubations performed with a large excess of unlabelled steroid), but can also be obtained by the use of correction terms [8,12].

In the case of aldosterone–receptor binding analysis, most of the authors, assuming a two site model, use the Scatchard method after correction for non-specificity [13–16]. The curve is empirically divided in two slopes corresponding to type I (mineralocorticoid) and type II (glucocorticoid) receptors; the parameters of the binding sites are then calculated by linear regression analysis of each slope.

This method was found to be very unprecise and

the purpose of the present paper is to report our studies of the aldosterone binding characteristics in rat kidney, using an in vitro experimental procedure [15] and recently developed mathematical and statistical methods including:

(i) Analysis of measurement dispersion.

(ii) Testing of increasingly complex models and calculation of parameter values with their confidence limits after best model determination.

(iii) Study of interexperimental variability.

2. Materials and methods

2.1. *Hormone binding assays*

Male Wistar rats (180–200 g) used for all experiments were adrenalectomized 2–5 days before sacrifice and maintained on normal saline ad libitum.

Animals were killed by aortic puncture, the kidneys removed and decapsulated after complete exsanguination. They were then minced with scissors and placed in 3 vol. ice-cold buffer (10 mM Tris-HCl, 1 mM EDTA, 10% v/v glycerol, pH 7.4; 1 mM dithiothreitol was added immediately before each experiment). After potter homogenization, the mixture was centrifuged at 700 × *g* for 10 min. Aliquots, 1 ml, of the resulting supernatant were then incubated with increasing concentrations (from 2×10^{-10} M to 5×10^{-7} M; 24 concentrations for each experiment) of [1,2–^{3}H]-aldosterone (spec. act. 52 Ci/mmol, Radiochemical Center, Amersham).

The solutions were centrifuged for 1 h at 30 000 × *g* and the supernatant was allowed to stand 1.5 h more. The total incubation period of 2.5 h was selected as in [15] and corresponded to the steady state. The total (T) cytosolic radioactivity was counted, bound (B) from free hormone were separated by the charcoal–dextran method [15] and counted; the unbound (U) hormone value was calculated by difference between T and B. Each T and B measurement was triplicated. Nine different binding assays were performed.

2.2. *Measurement dispersion*

The dispersion of the measurements was determined from similar experiments (see above) carried out at three different concentrations (1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M) of [^{3}H]aldosterone; for each concentration 24 incubations were performed.

2.3. *Radioactivity determination*

Aqueous samples were counted after addition of 10 ml Unisolve (Koch and Light) in a Tricarb 3380 scintillation spectrometer with a 24% efficiency. Correction for quenching was made.

2.4. *Statistical and numerical methods*

The experimental errors were assumed to be additive, uncorrelated, normally distributed and with a zero mean. Unknown variances were assumed to be B dependent. Analysis of error variances was determined from the experiments performed at three concentrations of [^{3}H] aldosterone (24 replicates for each concentration).

Four models of increasing complexity were tested:

One specific site

$$B = N \cdot U/K_d + U \quad (1)$$

One specific site + non-specific binding

$$B = N \cdot U/K_d + U + \beta \cdot U \quad (2)$$

Two specific sites

$$B = N_1 \cdot U/K_{d_1} + U + N_2 \cdot U/K_{d_2} + U \quad (3)$$

Two specific sites + non-specific binding

$$B = N_1 \cdot U/K_{d_1} + U + N_2 \cdot U/K_{d_2} + U + \beta \cdot U \quad (4)$$

The maximum likelihood approach [17] was attempted in order to test the model and to estimate the parameter values and their intervals of confidence (application of Rao-Cramer inequality [17]). According to the nature of experimental errors previously defined, a second order method [18] was used to minimize the following criterion:

$$C(\Theta) = \Sigma [B_{ci}(\Theta) - B_{mi}]^2/\sigma_i^2$$

where the parameter vector $\Theta = [N_1, N_2, K_{d_1}, K_{d_2}, \ldots\ldots]^T$ B_{mi} is the ith measured B, B_{ci}, the corresponding calculated value of B for a given mathematical model and σ the variance of B_{mi}.

The test was based on the value of the best criterion which is approximatively distributed as χ^2_{n-k} where n is the number of data and k, the number of parameters in the model [19]. Comparison of the criterion with tabulated χ^2 values for an α significance level permits the rejection of a model when the criterion $>\chi^2$.

Interexperimental distribution of the parameters was assumed to be Gaussian and uncorrelated. Maximum likelihood mean value and variance of the law was calculated from the different estimated parameter values and their confidence limits.

3. Results

3.1. *Experimental study of measurement dispersion*

The ratios of the standard deviation of B upon mean B values ($\sigma/\bar{B}$) are given in table 1.

The results show that $\sigma/\bar{B}$ appear to be constant for the three concentrations mentioned (T_1, T_2, T_3), implicating that σ depends on B values (and presumably on T values). For these reasons, a linear relationship could be assumed between σ and B ($\sigma = a \cdot B$), with $a = 0.04$.

It must be emphasized that this a value is applicable to a given binding assay only if B values corresponding to T_1, T_2 and T_3 are in the same range as in the measurement dispersion studies. This was found to be true for binding assays 1, 2 (see table 3).

Table 1
Values of B standard deviations (σ) ratio upon B mean values for three different concentrations of [^{3}H]aldosterone

[^{3}H]Aldosterone[a] (M)		$a = \sigma/\bar{B}$
T_1	1×10^{-9}	0.05
T_2	1×10^{-8}	0.03
T_3	1×10^{-7}	0.04

[a] 24 replicates for each concentration

3.2. *Test of increasing complexity models*

To test each model by the mean of the χ^2, we need to know the value of a in $\sigma = a \cdot B$. For this reason, the model was tested in the two binding assays 1, 2 where this value could be determined.

For other assays, the estimation was carried out assuming a linear relationship between σ and B with an unknown a value included in the parameter vector Θ.

The results of C minimization for four models in binding assays 1, 2 are shown in table 2.

Models (1) and (2) were rejected, models (3) and (4) were not; but when model (4) is considered, estimation of β gives a very small value (0.00013) with an interval of confidence including 0. For this reason, model 3 appeared to best fit the experimental data.

3.3. *Study of interexperimental variability*

Model (3) was choosen for the estimation of bind-

Table 2
Minimized criterion values and corresponding χ^2 values for an $\alpha = 0.05$ significance level

Model	Criterion value		χ^2 value
	assay 1	assay 2	
(1) $B = \dfrac{N \cdot U}{K_d + U}$	200	116	34
(2) $B = \dfrac{N \cdot U}{K_d + U} + \beta \cdot U$	170	95	33
(3) $B = \dfrac{N_1 \cdot U}{K_{d_1} + U} + \dfrac{N_2 \cdot U}{K_{d_2} + U}$	24	15	31
(4) $B = \dfrac{N_1 \cdot U}{K_{d_1} + U} \quad \dfrac{N_2 \cdot U}{K_{d_2} + U} + \beta \cdot U$	24	15	31

Table 3
Parameter values and confidence limits of aldosterone binding in rat kidney (homogenate 30 000 × *g* supernatant)

Assay	N_1		K_{d_1}	N_2		K_{d_2}
	M × 10^{11}	mol/mg protein × 10^{14}	M × 10^{9}	M × 10^{11}	mol/mg protein × 10^{14}	M × 10^{8}
1	34 ± 5	3.2 ± 0.5	2.15 ± 0.15	319 ± 31	30.4 ± 2.9	5.6 ± 1.3
2	34 ± 7	3.5 ± 0.7	3.00 ± 0.50	301 ± 24	30.7 ± 2.4	5.6 ± 1.4
3	48 ± 7	2.8 ± 0.5	1.58 ± 0.33	870 ± 71	50.0 ± 5.1	11 ± 1.8
4	50 ± 29	2.9 ± 1.7	2.00 ± 0.16	681 ± 64	40.0 ± 3.8	8.2 ± 1.8
5	46 ± 13	2.3 ± 0.7	1.60 ± 0.47	486 ± 42	24.8 ± 2.1	5.6 ± 1.3
6	79 ± 16	3.9 ± 0.8	2.45 ± 0.50	846 ± 178	42.3 ± 8.9	14 ± 5.2
7	45 ± 12	3.9 ± 1.0	1.74 ± 0.50	578 ± 60	50.7 ± 5.3	6.7 ± 1.6
8	35 ± 6	3.5 ± 0.6	1.16 ± 0.20	423 ± 23	41.9 ± 2.3	5.5 ± 0.9
9	25 ± 3	2.6 ± 0.3	0.63 ± 0.10	396 ± 15	41.2 ± 1.6	3.5 ± 0.3
Mean value[a] ± population SD	39 ± 8	3.2 ± 0.5	1.73 ± 0.58	520 ± 173	38.3 ± 7.7	6.1 ± 1.6

[a] Parameters mean value and interexperimental standard deviation (SD) was calculated by maximum likelihood estimation

ing parameters. Individual parameter values and their intervals of confidence for the nine binding assays studied are given in table 3. Interexperimental parameters (mean value and population standard deviation) is also mentioned.

A typical example of fitting of the experimental data by a curve simulated from model (3) is shown in fig.1.

4. Discussion

Interaction of [^{3}H]aldosterone with proteins of rat kidney was systematically studied by statistical methods. The first phase of this work was the determination of the best mathematical model describing aldosterone–protein interaction. In the resolution of such a problem, determination of measurement dispersion is of importance [8] since it greatly influences the identification criterion value and consequently the parameter value estimation as well as their intervals of confidence.

In binding experiments performed at increasing concentrations of [^{3}H]aldosterone, the bound fraction varies from 0.1 × 10^{-10} M to 50 × 10^{-10} M and it would have been inadequate to consider a unique standard deviation value for *B*. This would have been the same as minimizing an unweighted sum of squares

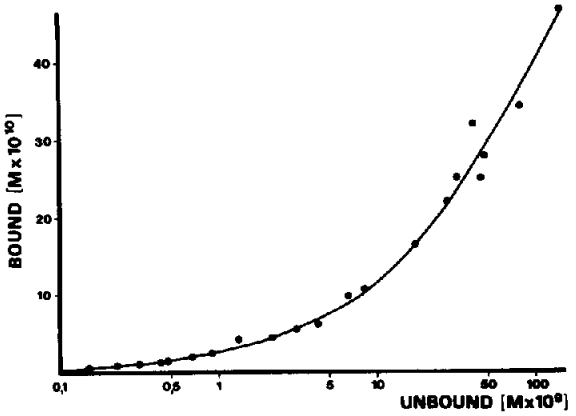

Fig.1. Plot of [^{3}H]aldosterone binding assay 7. Each point represents the experimental values; the curve was estimated by the computer from model (3).

criterion. Therefore dispersion was first studied in order to determine the relationship between standard deviation and B values. This relation was found to be linear and used for the ponderation of criterion.

Tests of increasingly complex models indicate that at least two saturable sites had to be considered to characterize the binding of aldosterone with its receptors; these results strongly back up previous hypothesis [13–16]. On the other hand, we observed (table 2) that the criterion C did not decrease when non-specific binding was tested together with two specific sites. This suggests that non-specific binding is not implicated in the range of aldosterone concentrations used.

Analysis of results presented in table 3 shows that the parameter estimation is very accurate in most of the experiments. This implies that experimental design is convenient, at least for the number of data considered. Interexperimental standard deviations are important factors since they represent the experimental variability and may be due to normal physiological differences. This must be carefully considered if one intends to study the binding characteristics of aldosterone receptor in pathological situations or if comparisons between animal species are to be made.

Our results demonstrate that the use of Scatchard method for determination of aldosterone binding characteristics is inadequate; in fact, it leads to an overestimated binding capacity and an underestimated affinity of mineralocorticoid sites, as suggested [13].

In conclusion, a simple, two step method to mathematically characterize aldosterone binding is described. This method could clearly be used for other types of protein–steroid interactions.

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