Absurd Trivial Errors in Scatchard Plot Analysis

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Summary: *Several types of widespread errors in performing Scatchard plot analysis resulting in fatal distortions of calculated affinities are surveyed more or less systematically. It appears that graphical analysis of non-linear Scathard plot "by eye" is almost impossible; and, moreover, results calculated by computer programs for affinity analysis often require additional "interpretation".*

Some of reported errors also definitely imply that data fabrication is quite common practice in this field.

Scatchard plot analysis is certainly one of the most popular procedures of data analysis in biomedical experimental research. Citation index gives over one thousand citations of original article (Scatchard G., 1949). Actually, about 70-80% of publications mention this procedure without citing anything, so, apparently, its using is reported by up to 5000 articles every year.

This procedure should be qualified as trivial algebraic manipulation: it is nothing more than redrawing data on a rather exotic coordinate plane. Yet the reality is that several absurd abuses completely depriving calculated result of any meaning are still extremely widespread in current scientific literature.

The main cause of there persistence is, of course, traditionally weak background of biomedical researchers in basic mathematics. Yet, actually, Scatchard procedure is not trivial; I clearly remember that I experienced serious discomfort dealing with it for quite a long time after first acquaintance. Perhaps, the more practical trouble was absence of a generally accepted description of this procedure and its pitfalls in literature. There are, perhaps, hundreds of theoretical papers on Scatchard plot analysis, but it is an extremely confusing collection consisting mostly of unimportant improvements, variations, extensions, etc.

Today articles on theory of Scatchard plot analysis are rare. Apparently, advent of simple computer alternatives for it brought peace. Yet, as it is shown below, some troubles do not disappear upon installing computer programs.

Basic Theory.

Affinity of ligand-binder reaction is usually determined by measuring concentration of bound ligand to a constant amount of binder at varying concentrations of ligand. This experiment produces the so called binding curve (also called saturation curve, adsorption isotherm, etc.) like one on Fig.1a.

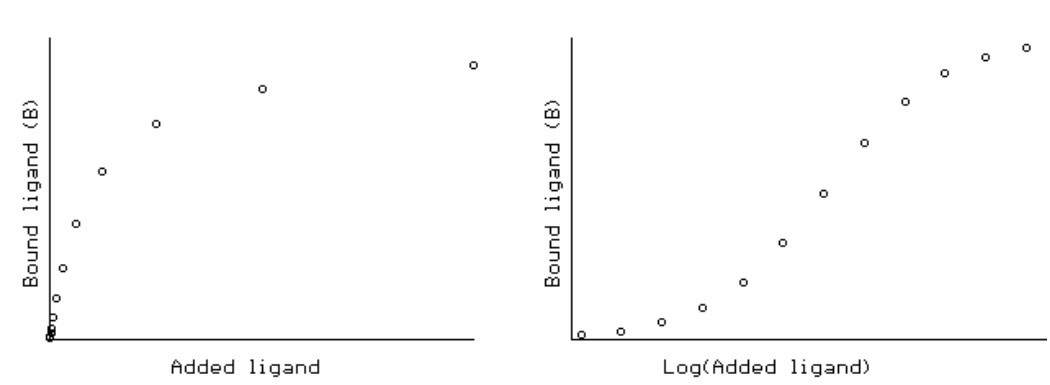

Fig 1a,b

It is worth to mention that if X-axis is in logarithmic scale, appearance of the curve changes to sigmoidal curve like in Fig.1b. Some may say that this metamorphosis is too trivial to mention it here at all. Yet, it was my personal experience of young years that after the first week spent at work I've suddenly found out that my own immediate boss (with about 10 years experience of drawing binding curves) does not believe me when I say him that curve on Fig.1a may be transformed to Fig.1b simply by putting X-axis in logarithmic scale. That boss was stupid well below average, but I have learned later that he is not alone who doesn't believe in this trick.

This story seems to be the right stuff to start with; all pitfalls depicted further are of approximately the same level of sophistication - something like “trivial folly”. Perhaps, the trouble with background signal looks slightly more serious, but, actually, it is also trivial.

If the binder is homogeneous and univalent, the binding curve is described by equation:

$$B = S_o*F/(1+K_o*F) \qquad [1]$$

where B is the bound ligand concentration; T - total (added) concn. of ligand; S_o - total concentration of binding sites, F - concn. of free ligand (i.e. F=T-B).

It can be seen from this equation that to recover affinity constant K_d from binding curve it is sufficient to find the free concentration F at the half-height of binding curve (i.e. where $B=S_o/2$). Obviously, visual determination of position of this point is defined just by several datapoints in the close vicinity of the center of binding curve. Therefore, if dataset is scant and noisy, large deviation of just one datapoint may lead to significant error in calculated K_d.

In order to include all other points into this process, the following procedure was proposed by Scatchard: the eqn.[1] is rewritten as:

$$B/F = S_o/K_d - B/K_d \qquad [2]$$

It may be understood from this form that if the binding curve is drawn on the coordinate plane B/F vs. B (it is Scatchard plot), then binding curve transforms into straight line with the slope giving affinity constant K_d. Visual determination of this slope is defined by all datapoints, so the precision of measuring K_d in this method will be improved.

Non-linear Scatchard plot.

If there are several populations of binding sites with different affinities to ligand then the binding curve is described by equation:

$$B= \Sigma S_i*F/(K_{di}+F) \qquad [3]$$

where S_i and K_{di} are concentrations and affinity constants of i-th population. It means that the binding curve on coordinate plane of Fig.1b (to be correct, on plane *Bound* vs. *Free*) is just a sum of all separate binding curves produced by all subpopulations presented. Unless difference in affinities of populations is very big (over 100-fold) the binding curve from such heterogeneous population become slightly more shallow than in the case of homogeneous binder while its shape remains essentially the same. So, it is usually rather difficult to say looking on the experimental data drawn on this coordinate plane whether it is produced by heterogeneous or homogeneous binder.

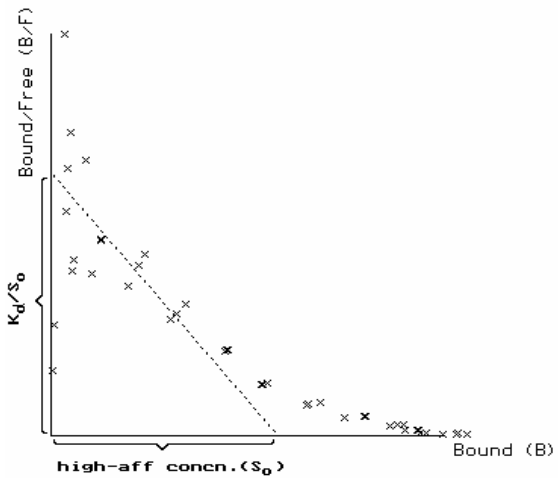

Fig 2

Drawing binding curve on Scatchard coordinates makes distinction more pronounced. If there are more than one population of binding sites, the Scatchard plot shows upward curvature like in Fig.2.

There was some fuss at the end of 70-ties about what is the correct way of graphical analyzing such curve (e.g. Norby J.G. et.al., 1980), yet I see little point in that controversy. Actually, different methods give almost the same value for "high-affinity" constant and the discord appears only in concentrations which are usually of no importance.

Here I will consider the following procedure correct: the regression line should be drawn through available datapoints and it should be extrapolated to the point of its intersection with Y-axis. The slope of tangent line drawn to this regression curve at this point gives the "high-affinity" constant and intersection of this line gives concentration of "high-affinity" binding sites (see Fig.3). It is important to say that thus defined affinity do not pretend to belong to any particular subpopulation, this is a kind of average constant given by equation (e.g: Gandolfi A. & Strom R.,1981):

$$K_{d\ h.a.} = \Sigma S_i * K_{di}^2 / \Sigma S_i * K_{di} \qquad [4]$$

where S_i and K_{di} are correspondingly concentrations and affinities of every i-th fraction of binding sites in the heterogeneous population.

Naturally, this "high-affinity" constant is equivalent to usual constant if the binder is homogeneous.

What is wrong

Though advantages of Scatchard plot analysis described above are quite appreciable, their practical importance should not be overestimated: perhaps, the less-precise determination affinity from the halfheight of binding curve may give about 2-fold extra-error in K_d which may be reduced by Scatchard plot analysis. But non-calculational factors dealt with conditions of experiment (i.e. those that distort the dataset itself: uncertainties with specific radioactivity, impure reagents, inaccurate diluting, etc.) have significantly more dramatic influence on error in K_d. So, this additional 2-fold error is not too important.

At the same time redrawing dataset on this extremely peculiar Scatchard coordinate plane - rather difficult job for biomedical researchers who usually are not great friends of basic arithmetic - poses a number of additional problems that frequently result in errors much more serious than wanted-for flimsy 2-fold decrease of error in K_d.

Actually, researchers in various fields who use Scatchard procedure analysis may have different goals and needs, yet the present work has slightly narrowed scope:

the pitfalls depicted further concern mostly determination of "high-affinity" constant. Their general property is that failure to escape any of them results in more than 10-fold error in K_d (or in "high-affinity" K_d if plot is curvilinear) and, more commonly, in qualitatively wrong judgments about heterogeneity of binder.

1. Obviously insufficient datasets.

As it was said above, determination of K_d requires knowing the slope of the binding curve at the point of its intersection with Y-axis on Scatchard plot. Yet the trouble is that binding curve always ends at some distance from this point (i.e. at Bound concentration bigger than nil). Therefore, the actual procedure is to draw regression line through available datapoints and to extrapolate it to the intersection with Y-axis. Quite naturally, the common situation is that dataset is insufficient to make such extrapolation, for example like in Fig.3.

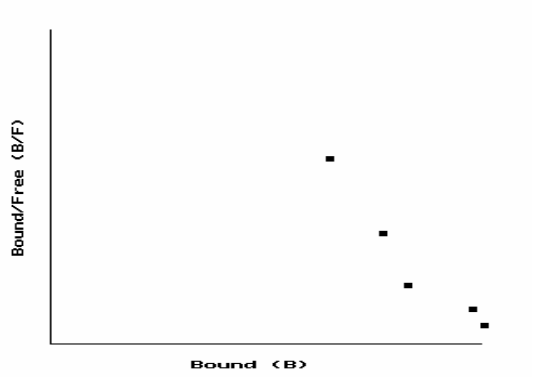

Fig.3

Frequent appearance of Scatchard plots that obviously fall into this category was quite explicitly reported by Irving Klotz (Klotz I.,1984) who seems to be one of the greatest authorities in affinity analysis. Yet similar cases still persist in experimental works (e.g. Buck E. et.al.,1992; Ahmad S.S. et.al.,1992; Schumacher C. et.al.,1992) and even in guides on affinity analysis (e.g. Berzofsky A.J. et.al., 1984) as examples of “nice-looking” plot.

To be correct, there is no sharp border between datasets which are sufficient to define intersection with Y-axis and those that are not. If available datapoints neatly lie on a straight line it may end much further from Y-axis than if they show some curvature like in Fig.3. The cited above papers contain "obviously" insufficient pictures, so trivial common sense may be quite effective to make distinction correctly.

In less certain cases it is advisable to use computer programs for affinity analysis. Interpretation of calculated result is the same as in determination of presence of background signal (see below): if there are no fractions in calculated affinity spectrum with very high affinities (i.e. with K_d smaller than the smallest concn. of *Free* ligand used in experiment) then your dataset is sufficient for determination of "high-affinity" constant.

2. Extensive scattering near Y-axis

It is also not a surprising news that Scatchard plot has a rather peculiar shape of data points’ scattering. For example, paper in Science (Munson P.J. & Rodbard D. , 1983) offers a “theoretical picture” explaining that scattering of points near Y-axis abruptly grows as *Bound* concns. become smaller.

Indeed, small concns. of *Bound* are measured with bigger relative error, and as the value of *Bound/Free* remains big at small *Bound* the visible scattering in *Bound/Free* is also big.

So, scattering of point near Y-axis is defined by relative error in *Bound* concns. and typical Scatchard plot should appear like Fig.2.

The trouble is that the number of articles containing Scatchard plots with such widening of error envelope is unnaturally rare. And, at the same time, ideal pictures with all points neatly lying on regression line throughout at least a 1000-fold span of Bound concns. are quite common.

Actually, accurate conducting of radioassay may ensure sufficiently small relative errors (below 5-10%) of measuring *Boun*d throughout 2-3 orders of magnitude of signal, in this case growth of scattering should appear closer to Y-axis, i.e. at *Bound* concentrations below 1% of maximal signal. So it is impossible to say that some particular binding curve is fabricated because it is too smooth. But it is more than strange that almost all publications contain such unnaturally perfect curves.

I think that it is the consequence of wrong prevailing opinion about the standard of a “nice-looking” Scatchard plot. Guides on binding analysis usually contain pictures showing only smooth lines without any datapoints. Naturally, such pictures produce an impression that experimental data should be uniformly scattered around it.

Of course, complying this wrong ideal requires data fabrication.

Most monstrously, some papers (e.g. Scheibe R.J. & Wagner J.A., 1992, Portolano S. et.al., 1993) published plots with points lying EXACTLY on the Y-axis. There is no hope to claim that such point lies “almost” on Y-axis when the next closest to Y-axis point lies at some detectable distance. Obviously, in this case there should be about 100-fold difference in *Bound* and, hence in *Free* concns. for these two points, what is unthinkable without special noting it in description of experimental procedure. So, presence of such “impossible” point is difficult to explain otherwise than by careless data fabrication.

There is also one more peculiarity in the mode of data points scattering which also give a sign to search fabricated data. The fact is that different measurements of *Bound* concns. made at the same *Free* concns. (it may be replicates or two different experiments) should lie approximately on the line drawn through the start of coordinates. If such points clearly lie in vertically-oriented groups (e.g. Scheibe R.J. & Wagner J.A., 1992) it is also a sure hint for presence of misconduct.

3. Inhibition curve

Instead of direct measuring of binding labeled ligand to binding sites the second standard procedure of measuring affinity K_d is to build the so called inhibition curve: i.e. to measure how different concentrations of unlabeled ligand inhibit binding of constant amount of labeled ligand to the binder. If experiment is conducted under the so called Cheng-Prusoff conditions (Cheng I. & Prusoff W.H.,1973), i.e. when free concentrations of ligands may be substituted by their total concentrations, the theory of inhibition experiment to homogeneous binder is rather simple. Inhibition curve is described by equation:

$$B=B_{max}*(1 - K_d/(K_d+T)) \qquad [5]$$

Where B_{max} is maximal signal on curve, K_d - affinity to unlabeled ligand. This equation means that the inhibition curve is just a binding isotherm turned upside down. So, to draw a Scatchard plot from inhibition curve it is necessary to take B_{max}-B instead of B, so the coordinate plane is $(B_{max}-B)/T$ vs. B_{max}-B.

But the fatal trouble with this procedure is that scattering of datapoints near Y-axis depicted in previous chapter becomes absolutely unbearable. Obviously, the error

in difference B_{max}-B grows as this difference itself decreases, so the Coeff. of variation grows absolutely unacceptably. For example, even the "ideal" inhibition curve with C.V. ~2% in the Bound concentration will result in C.V. ~40% in B_{max}-B when $B=0.95*B_{max}$. Therefore, ugly scattering on Scatchard plot when B_{max}-B becomes less then 5-10% of B_{max} is inevitable, in other words it is absolutely impossible to obtain "nice-looking" Scatchard plot following this procedure.

Actually, I am slightly uncertain about this section because the number of papers publishing ideal Scatchard plots derived from inhibition curves is too big to explain it by absent-minded data fabrication. I think there may be some alternative procedure of deriving something similar to Scatchard plot from inhibition curves (most likely, it may be dealt with documentation to LIGAND program by Munson P.J. & Rodbard D.(1980) which is not available to me) but I guess it should have some other name.

4. Background and non-specific signals.

There are two types of non-specific signals appearing in binding data. The standard terminology is not known to me, apparently it is absent, so I can't insist on their names given here.

The background signal is a constant addendum to all datapoints. In other words it is the signal at the "no ligand" vial appearing owing to radioactive dirt in measuring device, environmental background radioactivity or simply wrong setting of zero in device. It may be understood from eqn.(1) that constant addendum is equivalent to the presence of very high-affinity binding sites (i.e. $K_d<<F$) which are completely saturated throughout all binding experiment.

The nonspecific signal is due to the binding of ligand to the big amount of non-specific (i.e. very low-affinity) binding sites. Accordingly to equation (1) these binding sites ($K_d>>F$) will make a contribution to the binding curve equal to: $B_{non-sp}=Const*F$, or in other words a signal linearly growing with amount of added ligand.

Quite naturally, both background and non-specific signals may be experimentally measured and subtracted from summary signal. But the question is how dangerous is it if they are determined with some error and, therefore, some signal of either type remains in data analyzed following Scatchard procedure.

First, it may be definitely said that non-specific signal almost does not influence the calculated "high-affinity" constant. Until it is very big, there is little meaning in its accurate determination.

In contrast, the danger from background signal is catastrophic. To illustrate it the fig.4 draws the same binding curve with no additional background signal (solid line); with background signal equal to 1% of B_{max} (dot line) and to 5% of B_{max} (dashed line) at semilogarithmic and Scatchard coordinate planes. As it was said above, background signal is equivalent to the presence of very-high affinity binding, so the Scatchard plot shows it correspondingly. Obviously, if some background signal is presented in data, bound concentration never approaches zero and, therefore, the left section of Scatchard curve has a vertical asymptotic line intersecting X-axis at the concn. equal to the magnitude of background signal. If it is big it's presence may be detected by eye, this sort of published pictures is quite widespread (e.g. Hisabori T. et.al.,1992). Yet, if background signal is small and no vertical asymptotic line is detectable at the plot, it becomes practically impossible to distinguish visually background signal from true high-affinity binding.

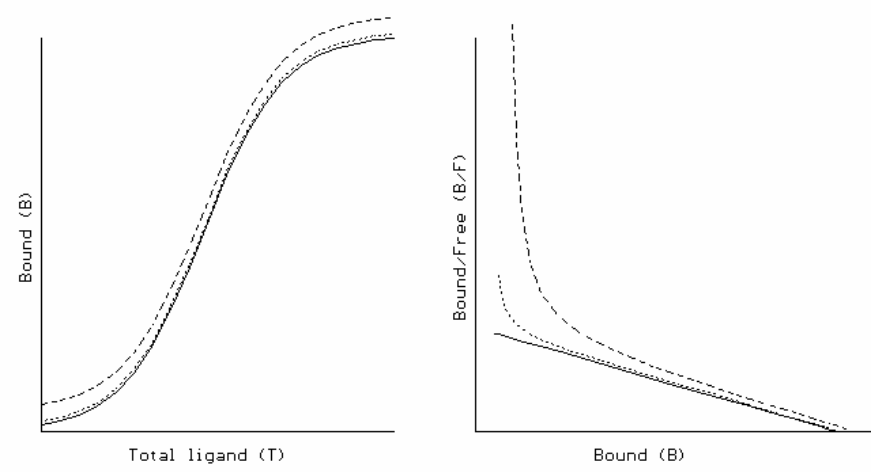

Fig 4

I think, it's a fatal pitfall; owing to it graphical analysis of Scatchard plot may be considered reliable only if there are no background signal at all or if the Scatchard plot is clearly linear. Otherwise, as you can see it on the left panel of Fig.4, even 1% addendum results in a pretty looking artifactually "heterogeneous" Scatchard curve.

To some extent this pitfall may be escaped using computer programs for affinity analysis. It may be both affinity spectrum approaches (e.g. Tobler & Engle,1983; Yuryev D.K.,1991) or multisite model programs (e.g. Munson P.J. & Rodbard,1980); yet in the second case the program should be run at least in 3-site mode. If some background signal is presented, either of these programs will give a subpopulation in calculated affinity distribution with "too high" affinity - with K_d smaller than the smallest Free ligand concn. used in experiment. Actually, running LIGAND in two-site mode may also give a "too-high" high-affinity constant and this sort of error is also quite widespread (e.g. Chadwick C.C. et.al.1992).

In this way background signal at least may be identified. Then it may be subtracted from analyzed data and, if necessary, the correct value of "high-affinity" constant may be estimated, but this procedure seems to be not quite reliable: the preferable way is to avoid dangers of background signal experimentally.

References

Ahmad S.S. et.al. (1992) - J.Biol.Chem., 267:8571-6.
Berzofsky A.J. et.al. (1984) - p.595, in: Fundamental Immunology. (ed.W.E.Paul), Raven Press, NY, 1989).
Buck E. et.al. (1992) - J.Biol.Chem., 267:23560-7.
Chadwick C.C. et.al. (1992) - J.Biol.Chem., 267:3473-81.
Cheng Y. & Prusoff W.H. (1973) - Biochem.Pharmacol.,22:3099-108.
Gandolfi A, Strom R. (1981) - J.Theor.Biol. 92:57-84.
Hisabori T. et.al. (1992) - J.Biol.Chem., 267:4551-56.
Klotz I.M. (1985)- Quarterly Rev.Biophys. 18(3):227-59.
Munson P.J., Rodbard D. (1980) - Anal.Biochem. 107:220-39.
Munson P.J. & Rodbard D. (1983) - Science, 220:979-81.
Norby J.G. et.al. (1980) - Anal.Biochem., 102:318-20.
Portolano S. et.al. (1993) - J.Immunol., 150:880-7.
Scatchard G. (1948) - Ann.N.Y.Acad.Sci., 51:660-72.
Scheibe R.J., Wagner J.A. (1992) - J.Biol.Chem., 267:17611-16.
Schumacher C. et.al. (1992) - PNAS USA, 89:10542-6.
Tobler H.J. & Engle G. (1983) - Naunyn-Scmied.Arch.Pharmacol., 322:183-192.
Yuryev D.K. (1991) - J.Immunol.Meth., 139:297.