A4.8 *Fitting relative potencies and the Schild equation*

A4.8.1. Constraining fits to sets of curves

It is often necessary to deal with more than one curve at a time. Typical examples are (1) sets of parallel log(concentration)-response curves designed to estimate the relative potencies of a series of agonists, or for biological assay of an unknown concentration; (2) sets of parallel log(concentration)-response curves obtained by adding successively greater concentrations of a competitive antagonist; (3) sets of current-voltage curves obtained in the presence of different concentrations of a channel blocking drug.

In cases such as these, it is always possible to fit each curve separately. More often, though, it will be desirable to put some sort of constraint on the fits. For example, in the case of sets of log(concentration)-response curves, it may be desirable to fit all curves with a common maximum value, the same for all curves. It may also be desirable to fit curves that are constrained to be parallel. In the case of experiments with competitive antagonists, it may be desirable to add the further constraint that the curves are not only have the same maximum and are parallel, but also have the spacing dictated by the Schild equation (see Chapter 3), as illustrated below. These procedures are illustrated below. In the case of sets of current-voltage curves it may be desirable to constrain all curves to having the same reversal potential, and/or to constrain the curves to the spacing dictated by a postulated mechanism for channel block. Unfortunately, although commercial programs may perform adequately for fitting single curves, they are mostly incapable of doing standard jobs of the sort just described.

A4.8.2 An example of fitting results with a competitive antagonist.

Estimating dose ratios from a set of concentration-response curves

The fitting of sets of log(concentration)-response curves will be illustrated by results obtained by Black & Shankley for the inhibition by atropine of the change in pH elicited by the muscarinic agonist, 5-methylfurmethide, in the isolated lumen-perfused mouse stomach preparation. Fig A4.10 shows the log(concentration)-response curves for the agonist alone, and in the presence of six different atropine concentrations (concentration is marked on each curve). Each point is the mean of 1 to 5 observations, and the observed standard deviations of the means (see section A4.2). Each curve has been fitted separately, in an entirely empirical way, with the Hill equation, using weighted least squares (the weights being taken from the observed standard deviations; see eq. A4.4.4). It can be seen that the curves are not precisely parallel: the estimates of n_H vary from 1.65 (atropine concentration 1 μM) to 2.74 (zero atropine concentration). The curves also clearly differ in their maximum responses (they were determined on 6 different preparations on 7 different days, so there is no reason why the maxima should be the same).
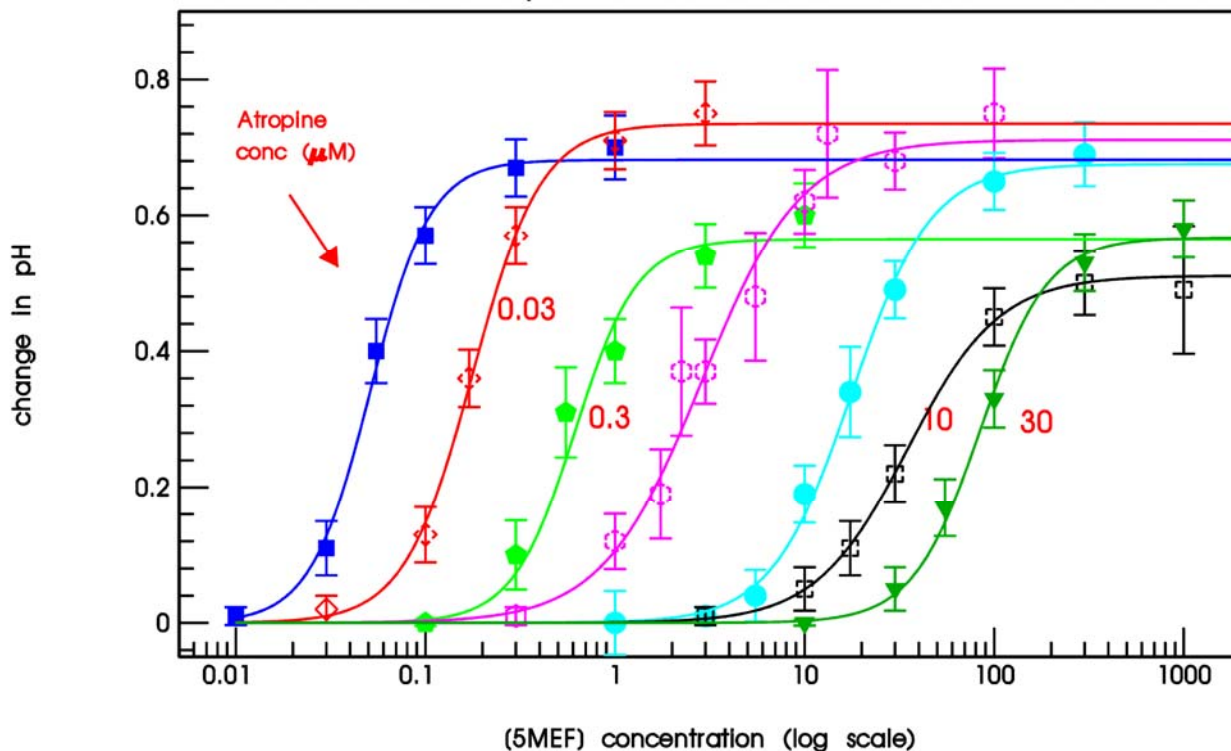
Fig A4.10

Experimental results of Black & Shankley, as described in the text. The atropine concentration for each concentration-response curve is marked on the curve. The error bars show the standard deviations of the mean responses. The solid lines show the fit to each curve separately of the Hill equation . The fits were done by weighted least squares, using smoothed versions of the standard deviations shown here (see text and Fig A4.11)

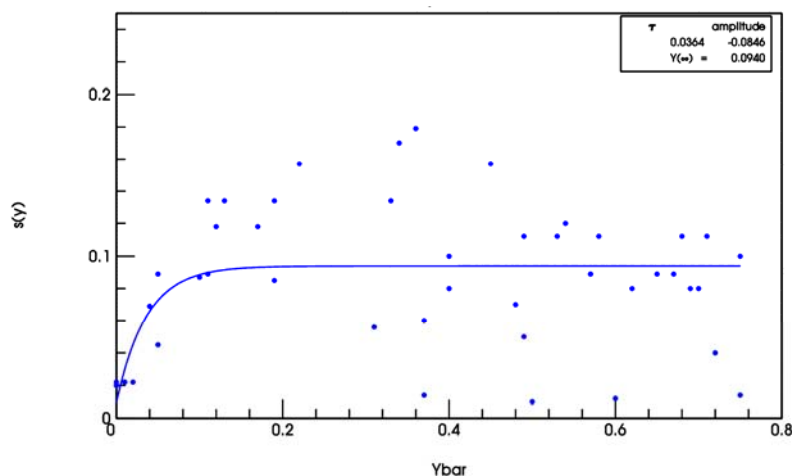| τ	amplitude
0.0364	-0.0846
$Y(\infty) =$	0.0940

Figure A4.11

Plot of the standard deviation of an observation against the mean response observed at the concentration. The values were found by multiplying the standard deviations of the means, shown in Fig A4.12, by the square root of the number of observations in the mean. A rough line has been drawn through the points to allow smoothed values to be read off

When fitting these curves, the weights were not taken from the observed standard deviations of the means (those shown on the graph). These standard deviations of the observations are based on quite small numbers of replicates so they are quite variable. Before fitting, the values are therefore smoothed, as suggested in section A4.3.2. Fig A4.11 shows a plot of the observed standard deviation of an observation ($\sqrt{n}$ times the standard deviation of the mean), plotted against the mean response to which it is attached. The standard deviations are indeed quite scattered, but it is clear that they are small for the smallest responses, and then rise to a more or less constant value of about 0.09. A rough curve was drawn through the points and smoothed values for the standard deviations were read off from it.; these were converted back to standard deviations of the means, by dividing by the appropriate $\sqrt{n}$, and these values were used to calculate the weights for the subsequent fits.

There are many ways in which these results could be analysed. We shall proceed by first taking the separate Y_{max} values for each curve, as estimated by the fits in Fig A4.10, and divided

each response for the curve by that value, so the maximum response is normalised to 1 for each curve. The standard deviations for each point are also scaled by the same Y_{max} value (this is, strictly
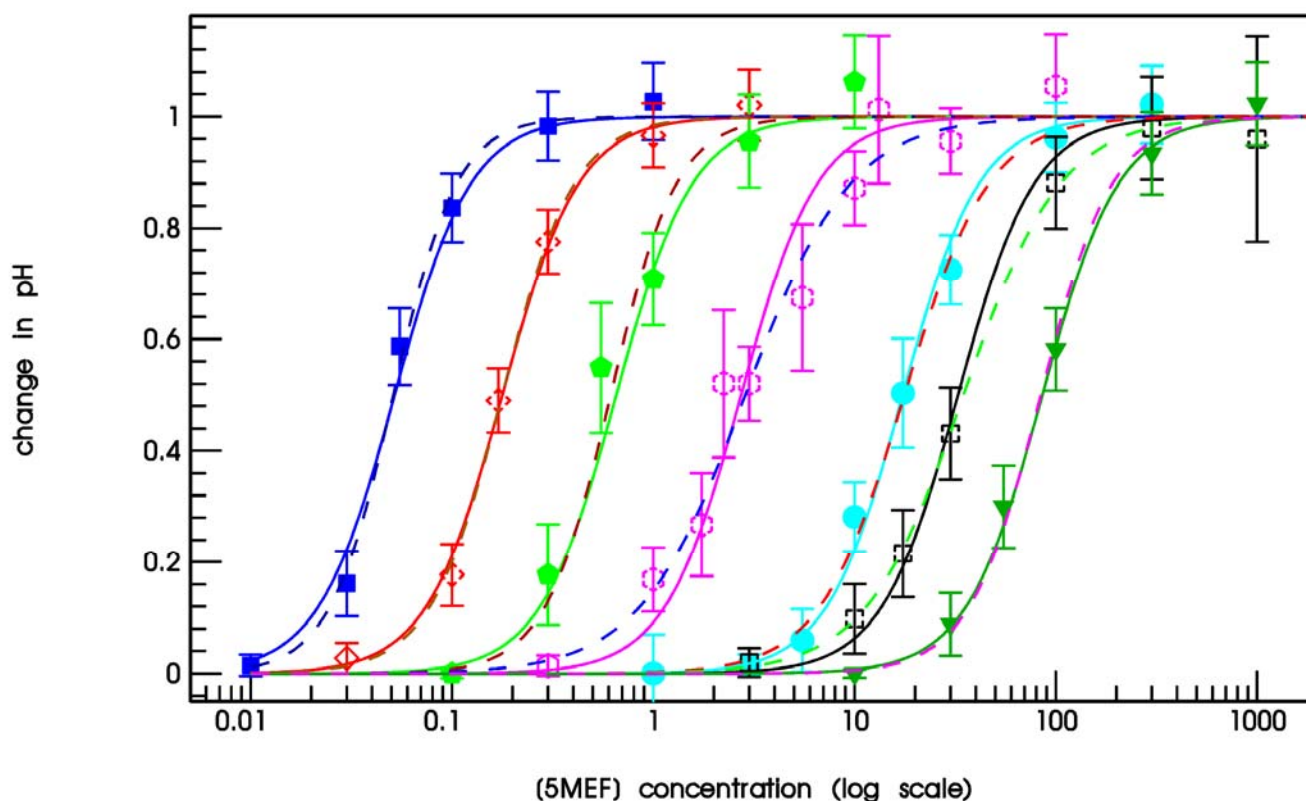

Fig A4.12
Experimental results of Black & Shankley, with responses (and smoothed errors) divided by the maximum response, as fitted in Fig A4.10, to normalise the maximum response to 1 for each curve. The dashed lines show separate fits of with Hill equations to each curve, with the maximum response fixed at 1. The solid lines show a fit to the set of curves with a common n_H estimate, so the curves are constrained to be parallel.

speaking, proper only if the Y_{max} values have no error, which is certainly not true). The result is the set of normalised log(concentration)-response curves shown in Fig A4.12. The error bars in this graph are the scaled and smoothed standard deviations of the means, found as above. These curves were fitted by Hill equations with the maximum response now fixed at 1 for all curves, and the results of this fit are shown as dashed lines in Fig A4.12. They are still not parallel. (An alternative is to fit all curves with a common maximum; when this is done the common maximum comes out to be 0.993, very close to 1, as expected.) Since the curves are not exactly parallel, they still do not define unique dose-ratios for the analysis of the antagonism. One way to deal with it is to determine the dose-ratios at some arbitrary response level (commonly 50% of maximum), but the results will depend on what level is chosen. This is clearly undesirable.

A better method is to fit a set of log(concentration)-response curves that are constrained to be parallel. These are the solid curve is Fig 4.12, which are exactly parallel. This was done by again fitting Hill equations. This equation has three parameters (Y_{max}, EC_{50} and n_H), so the initial separate fits of the seven curves in Fig A4.10 involved fitting 21 parameters. The separate fits (dashed lines in Fig A4.12) all have the maxima fixed at 1, so 14 parameters are fitted (seven EC_{50} and seven n_H values). The solid lines in Fig A4.12 all have the same slope so only eight parameters are fitted (seven EC_{50} values and one, common, n_H value, or, equivalently, one EC_{50} value for the control curve, six dose-ratios and one n_H value). The common slope came out as $n_H = 2.29 \pm 0.14$. When the curves are constrained to be parallel, the fit will, of course, not be as good as when no

such constraint is applied. A statistical test can be done to see whether the curves are 'significantly non-parallel.

The Schild plot: fitting a straight line

The dose-ratios obtained from the fit of the solid lines in Fig A4.12 are plotted as a Schild plot in Fig A4.13. The error bars shown on this plot were obtained as

$$s(\log(r)) \approx \frac{s(r)}{2.303r} \ , \qquad\qquad\qquad (A4.8.1)$$

where $s(r)$ is the approximate standard deviation of the dose ratio, r, found from the fit in Fig. A4.12 (see, for example, Colquhoun, 1971). The error bars for $\log(r - 1)$ are see to be roughly constant at about $\pm$ 0.05 - 0.06 $\log_{10}$ units, showing that the dose ratios themselves have a roughly constant coefficient of variation (of 10.5-14%). A straight line was fitted to the points in Fig A4.13, using the weights shown, and its slope came out to be 0.983. If the error is calculated on the basis of the weights (as discussed above), the slope is 0.983 $\pm$ 0.021. If on, the other hand, the error estimate is based on the deviations of the points from the fitted line (the *residuals*), the slope is 0.983 $\pm$ 0.072. In neither case is the slope is 'significantly different' from the value of 1 predicted by the Schild equation. The larger error found from residuals is not surprising. Although the Schild plot looks reasonably linear, two of the points are quite a long way from the fitted line (relative to their error bars), and these points will make the error estimated from residuals bigger than expected.
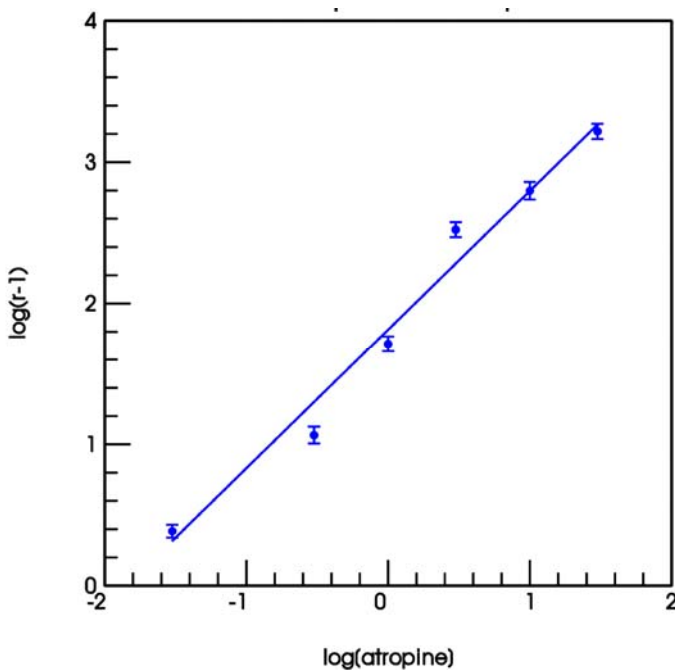

Fig A4.13
Fit of a straight line to the dose ratios obtained by the fit shown in Fig A4.12. The log(r −1) values were equally weighted
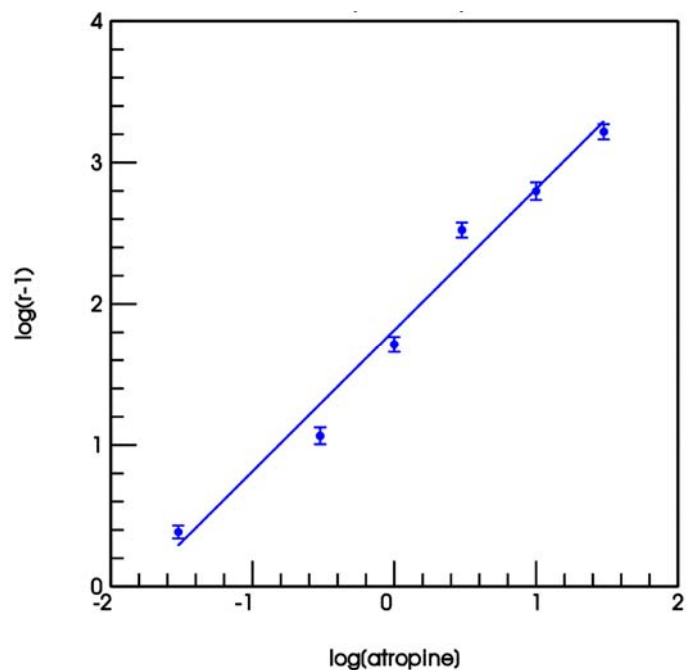
Fig A4.14
Fit of the Schild equation to the dose ratios obtained by the fit shown in Fig A4.12. The slope is fixed as 1. The log(r −1) values were equally weighted

Fitting the Schild plot equation to dose ratios

The result of fitting a line to the Schild *plot* was that the data are consistent with the Schild equation. This means that it is reasonable to go ahead and *fit the Schild equation to the dose ratios*. The straight line we have just fitted was not a fit of the Schild equation, because its slope was not (exactly) 1. When we wish to test any physical theory, the standard procedure is to fit to the data the equation predicted by that theory. In this case the equation to be fitted is the Schild equation, $r = 1 + x/K_B$. This equation has only one free parameter, the equilibrium constant for the binding of the antagonist, K_B. We must, therefore, re-fit the data in Fig. A4.13 with the Schild equation, in order to estimate this one parameter. This is shown in Fig A4.14, in which the slope is fixed at 1. From this fit we find the midpoint (the value of Y at $x =0$) to be 1.81 ± 0.022 (with errors based on the error bars). This is the negative logarithm of K_B so we estimate K_B as 0.0154 μM, or 15.4 nM. The error for $s(\log(K_B)) = 0.022$ implies that K_B itself has a coefficient of variation of about 2.303 $s(\log(K_B)) \approx 5\%$. This, of course implies unequal intervals above and below 15.4 nM. An approximate standard deviation for the estimate of K_B can be found as 2.303 K_B $s(\log(K_B)) = 0.77$. Thus the final result of this analysis can be expressed as $K_B = 15.4 \pm 0.77$ nM.

In this particular case, the fit of the Schild equation looks very similar to the free fit because the slope was close to 1 anyway, but this will not always be the case. If, for example, the free fit had given a slope of 0.85 ± 0.1, we might have decided to proceed with the Schild analysis on the grounds that the slope was not 'significantly' different from 1 (though we should have to bear in mind that the slope is not 'significantly' different from 0.7 either). In this case the fit of the Schild equation would give a substantially different of K_B from that which would be obtained if K_B were (erroneously) estimated from the free fit.

Direct fit of the Schild equation to the original observations

An alternative method for analysis of this sort of experiment was proposed by Waud (1975). This method bypasses the Schild plot altogether, and fits the Schild equation directly to the original concentration-response curves. In order to do this it is, of course, necessary to postulate a form for these curves, and we have already found that the Hill equation provides an adequate empirical description of them. The entire set of curves in Fig. A4.12 is fitted simultaneously with only three free parameters, the EC_{50} for the control curve, a common Hill slope, and K_B. In other words the curves are constrained not only to be parallel, and to have a common maximum (fixed at 1), but also to have a spacing that is dictated by the Schild equation.

The result of this fitting is shown in Fig. A4.15. There is, of course, no point in doing a Schild plot on the dose ratios read from the curves, because it will inevitably have a slope of exactly 1 as a result of the way the fitting was done. This fit gave an estimate of $K_B = 14.8 \pm 1.41$ nM. In this case the approximate standard deviation for K_B is found in the fitting process as described above. Because K_B is one of the parameters being fitted, likelihood intervals for it can be found as above. The 0.5 unit intervals (the equivalent of one standard deviation) come out as $14.8 - 1.35$ nM to $14.8 + 1.41$ nM, and the 95% likelihood interval for K_B is $14.8 - 2.58$ nM to $14.8 + 3.14$ nM.
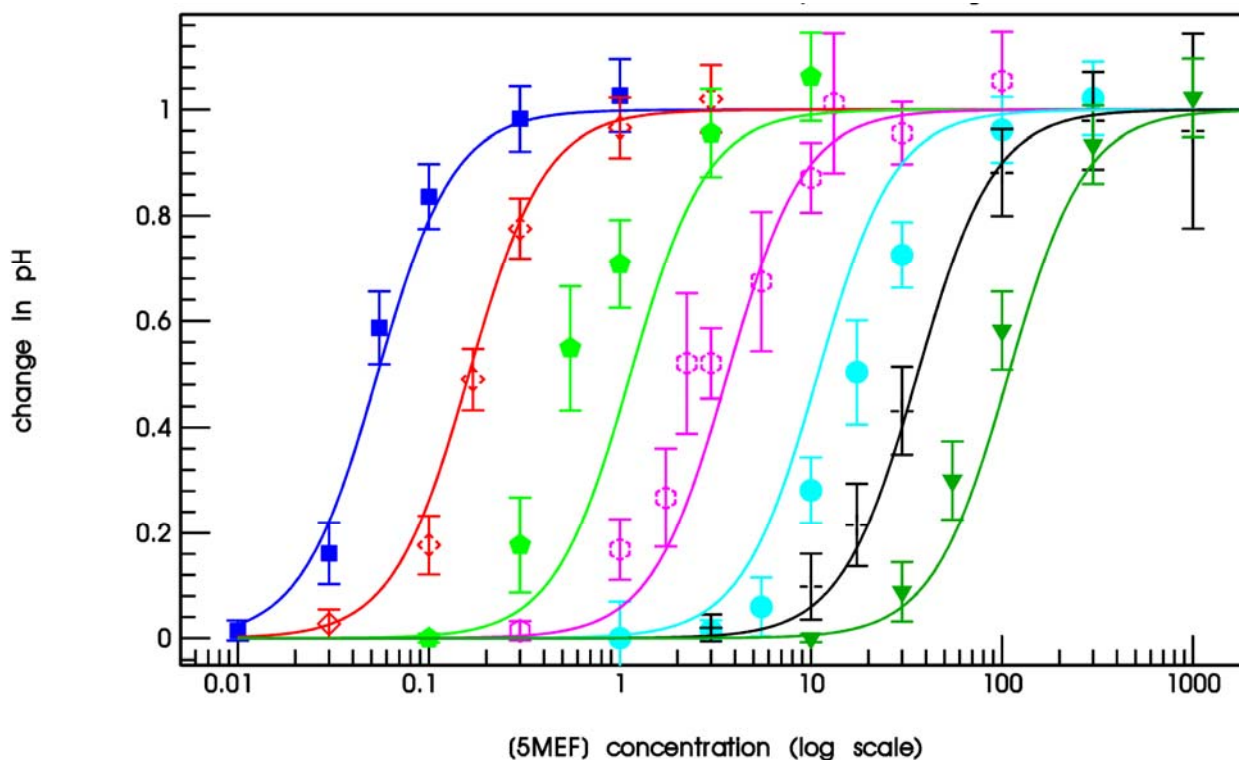
Fig A4.15

Experimental results of Black & Shankley, showing a direct fit of the Schild equation to the original data (supposing, as before, that a Hill equation can describe the shape of the curves adequately). The error bars showed the smoothed errors that were used to weight the fit, as in Fig A4.12.

It may be, though, that we should not be calculating a value of K_B at all from this fit, because two of the curves fit the data quite badly (those for 0.3 and 3 μM atropine). These are exactly the two points which deviate noticeably from the Schild plot in Fig A4.14, but the deviations are much more obvious when they are shown on the original data. It is well known that logarithmic scales can disguise a multitude of sins, and this is a good example. The Schild plot looks fairly satisfactory, but the display of the same deviations from the Schild equation shown in Fig A4.15 look quite unsatisfactory.

Pharmacological postscript

It is very salutary to note that all of this fancy analysis turns out to be quite fallacious in the present example. In fact K_B is seriously over-estimated in this particular preparation, probably because of loss of the antagonist into the gastric secretion (Black & Shankley, 1985, *Brit. J. Pharm.*, 86, 601-607). In fact the K_B for atropine is close to 1 nM for all five known receptor subtypes. If we were to interpret the value of 15 nM found here as being evidence for a new subtype we would (almost certainly) be wrong, for reasons that have nothing to do with curve fitting.