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Letter to the Editor

Determination of ligand–macromolecule binding parameters and the method of Hummel and Dreyer

Sir,

A recent article in this journal has demonstrated the usefulness of high-performance liquid chromatography (HPLC) in the study of ligand binding by a macromolecule[1]. The application of the method of Hummel and Dreyer[2] to HPLC appears very promising in the study of these interactions. For this reason, the following suggestions are made in an effort to improve this method further.

In the method of Hummel and Dreyer[2] a size exclusion column is equilibrated with a solution of ligand of concentration [A]. The macromolecule is dissolved in this ligand solution, applied to the column and eluted with the same solution of ligand. During this elution a peak representing bound ligand is observed at the void volume of the column. At some point after this peak a trough is observed which also represents bound ligand. The amount of ligand bound may be determined from the area of either the peak or trough. More precise determination of the extent of binding is possible using a plot of the area of the ligand trough (positive or negative) *versus* the excess moles of ligand (relative to [A]) added to a constant amount of macromolecule. This plot is used to determine the excess of ligand at zero trough area which equals the amount of ligand bound.

In the application of the method of Hummel and Dreyer[2] to HPLC the graphical method of determining the moles of ligand bound was utilized[1]. Two requirements which must be met if the graphical method is valid were not discussed. The first requirement is simply that the area of the peak at the void volume of the column must be constant for the various amounts of excess moles of ligand. The second requirement is based on the following equation:

$$a = M - A - B \qquad (1)$$

where

a = trough area (positive or negative) in moles of ligand,
M = total moles of ligand in the injected sample,
A = moles of free ligand = s[A],
B = moles of bound ligand,
s = volume of sample injected.

As expected, when $M = A, a = -B$ (since the trough area is negative) and when $M = A + B, a = 0$. Eqn. 1 may be stated in terms of the excess moles of ligand added, E since $E = M - A$. Substitution of the latter equality into eqn. 1 yields:

$$a = E - B \qquad (2)$$

As expected, when $a = 0, E = B$ and when $E = 0, a = -B$. Based on eqn. 2, the plot of a *versus* E should be linear with a slope of 1, an abscissa intercept of B and

an ordinate intercept of $-B$ (within experimental error) if the method is valid.

If the area of the trough is plotted in arbitrary units, a' rather than a, the plot of a' *versus* E will be linear if the method is valid and if a' is a linear function of a. When $a' = ca$, where c is a constant, the slope of the plot of a' *versus* E will equal c if the method is valid. Again, the abscissa intercept will equal B, but the ordinate intercept will equal $-cB$. To determine if the plot of a' *versus* E is valid the value of c must be known. If values of a rather than a' are determined, eqn. 1 may be used to obtain a value of B for each data point rather than the single value of B normally obtained through a plot of a' *versus* E. In this way the mean and standard deviation of B may be easily determined.

It has previously been shown that the concentration of the ligand in the injected solution must be corrected for the volume occupied by the macromolecule and the presence of water in the macromolecule[3]. The following correction for M is suggested when a known quantity of macromolecule in a volumetric flask is brought to volume with ligand solution.

$$M = s[\mathrm{L}](1 - P[w + \bar{V}(1 - w)]) \quad (3)$$

where

[L] = molar concentration of ligand solution added,
P = concentration of macromolecule (g/ml),
w = fractional water content of the macromolecule,
$\bar{V}$ = partial specific volume of the macromolecule (ml/g).

Finally, it is important in the study of ligand–macromolecule interactions to determine if the value of r, the moles of ligand bound per mole of macromolecule, is dependent on the concentration of macromolecule at a given value of [A] (ref. 4). This is especially important in the Hummel and Dreyer method where the macromolecule solution is always diluted during chromatography. If the interaction is macromolecule concentration dependent the value of r will vary across the macromolecule peak eluted. In these cases values of r should be calculated for various values of s at constant values of [A] and the macromolecule concentration since dilution of the macromolecule decreases as s increases[5]. If the macromolecule is significantly diluted during chromatography but r does not vary with s either the decrease in dilution with increase in s is insignificant or the ligand trough is formed before the macromolecule is significantly diluted. If r does vary with s, perhaps the value of r at the initial macromolecule concentration can be estimated as the ordinate intercept of a plot of r *versus* $1/s$.

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