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Hummel-Dreyer method in high-performance liquid chromatography for the determination of drug-protein binding parameters

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ABSTRACT

Two types of Hummel-Dreyer elution pattern were developed, one with a positive peak followed by a negative peak and the other with two positive peaks. The evaluation of the area of the second peak, whether positive or negative, makes it possible to determine the value of L_b (the number of moles of the ligand bound to macromolecule) or $[L]_b$ (the concentration of the ligand bound to macromolecule). Three techniques were employed for the evaluation of the area: planimeter, geometry and integrator. Planimeter and geometry can be used for both types of elution profiles and for both external calibration and internal calibration. The integrator can only be used for the second type of elution profile, namely two positive peaks and hence, can only be used in conjunction with internal calibration. The results in terms of the two binding parameters, n (the number of binding sites) and k (the affinity constant) in binding equilibrium for L-tryptophan–bovine serum albumin system were compared. Realizing that uncertainty involved is large for the binding studies in any experimental method for the binding studies, we believe that any of the five combinations (among external calibration, internal calibration, planimeter reading, geometry reading and integrator reading) would lead to a reasonably accurate result.

INTRODUCTION

The Hummel-Dreyer method [1] in high-performance liquid chromatography (HPLC) has been demonstrated as a useful tool for the determination of drug-protein binding parameters [2,3]. The chromatogram shows a positive peak, which represents the drug-protein complex, followed by a negative peak, which represents the elution volume (or time) of the drug. It is the negative peak that provides the information about the amount of the drug bound to the protein. The evaluation of the area of the negative peak makes it possible to calculate $[L]_b$ or L_b, where $[L]_b$ is the concentration of bound drug in M and L_b is the absolute quantity of bound drug in mol. Experimentally, $[L]_b$ is obtained with external calibration technique and L_b is obtained with an internal calibration technique [4,5].

However, a Hummel-Dreyer pattern of elution profile does not have to be in the form of a positive peak followed by a negative peak. Instead, we can have two positive peaks [6–8]. This is done by adjusting the concentration of drug in the protein sample solution in a reasonable excess over that in the eluent.

Thus, there are two different patterns to run a Hummel-Dreyer experiment. In either pattern, the evaluation of the area of the second peak is a time-consuming process. So far there is no way to tell which pattern is superior over the other. It all depends on which pattern is easy to be dealt

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with in the evaluation of the area of the second peak. In this paper we compare three different techniques for the evaluation of the area of the second peak: planimeter, integrator and geometry. The first two techniques are not new, whereas the third one perhaps is the first time to be described in detail in HPLC. Since the evaluation of the area of a curve is a common problem in many fields such as diffusion and sedimentation, we believe the techniques we learn in HPLC could be extended to the other experimental fields and vice versa. As a test system, we choose L-tryptophan–bovine serum albumin (BSA) binding for study. Not only the subject is of great interest in biochemistry and pharmacology [9–11], but also data obtained from different experimental methods, e.g. equilibrium dialysis, is abundant in literature. Hence, it is relatively easy to asses the validity of a new method to be introduced.

EXPERIMENTAL

Materials

L-Tryptophan (lot No. 6766) was obtained from Mann Research Lab. (New York, NY, USA) and BSA (lot No. 29F9315) was a product of Sigma. Sodium hydrogenphosphate and sodium dihydrogenphosphate were reagent grade of J.T. Baker. All the chemicals were used as received. Glass-distilled water was used in all experiments.

Preparation of solutions

The procedure for the preparation of solutions was similar to the one described in previous publications [4,5]. Table I describes the preparation of the mobile phase of L-tryptophan solutions and Table II describes the preparation of protein-tryptophan samples to be injected onto the column. In the preparation of mobile phase, we diluted the stock solution (77.2 μM) into five different concentrations. Those mobile phases selected in Table I are best for the internal calibration measurement. Samples A, B, C, D, E in Table II were prepared for the internal calibration technique and F and G were prepared for the external calibration technique.

TABLE I

PREPARATION OF MOBILE PHASE

Stock solution: L-tryptophan, 77.2 μM; phosphate buffer 0.05 *M*, pH 7.4.

Mobile phase	L-Trp (ml)	Buffer (ml)	Dilution factor	$[L]_f$ (μM)
1	200	1300	0.14	10.3
2	400	1100	0.28	20.6
3	500	1000	0.35	25.7
4	600	900	0.42	30.9
5	800	700	0.56	41.2

Chromatography

The size-exclusion chromatography was carried out at room temperature (near 25°C) with one Waters Assoc. pump A-6000, an injector UK-2, an UV variable-wavelength detector, a recorder and a Hewlett-Packard integrator 3965. The column used was Waters I-125 (30 cm x 7.8 mm I.D., particle size 37-53 μm). This column is not an ideal one, but it is easily accessible.

Evaluation of the peak area

As mentioned before, we used three entirely different methods for the estimation of the second peak in the chromatogram.

Planimeter. Planimetry is the oldest and classi-

TABLE II

PREPARATION OF PROTEIN-TRYPTOPHAN SAMPLES

Stock solutions: L-tryptophan, 77.2 μM; BSA, 495 μM; phosphate buffer 0.05 M, pH 7.4.

Sample	BSA solution (ml)	L-Trp solution (ml)	Buffer (ml)
A	2.0	9.0	4.0
B	2.0	10.0	3.0
C	2.0	11.0	2.0
D	2.0	12.0	1.0
E	2.0	13.0	0
F	2.0	0	13.0
G	0	0	15.0

cal technique. It is reasonably reliable and it can be applied to any shape of the graph. But it is tedious and it requires the techniques of the user. In previous publications [4,5] this technique was used exclusively for the evaluation of area.

The integrator. The integrator available to us such as Hewlett-Packard 3395 cannot be used for the evaluation of the area of a negative peak. For this reason it cannot be used for the external calibration method to determine the binding parameters. It can only be used for the internal calibration method with a two-positive-peak elution profile. However, since the data of the area are automatically printed out, the integrator is the best tool to save time in reading the area.

Affine geometry. We believe this is the first time affine geometry is introduced to estimate the area of a peak in scientific research. For this reason, we shall describe the geometry briefly. According to affine geometry, for any triangle ABC with the coordinates of three vertices known, namely, $A(x_1,y_1)$, $B(x_2,y_2)$ and $C(x_3,y_3)$, the area can be accurately calculated in terms of the following determinant:

$$A = 1/2 \begin{vmatrix} x_1 & Y2 & 1 \\ x_2 & y_2 & 1 \\ x_3 & Y3 & 1 \end{vmatrix}$$

This theorem has been rigorously proved [12]. In a HPLC chromatogram, the peak or trough is often shown almost as a triangle. This is particularly so when the column is new or the number of theoretical plates is high. If the units of x and y coordinates are cm, then the area is in cm^2; if in inch (1 in. = 2.54 cm), then in.‘. We can even choose any arbitrary units as is the case with integrator, since the final scale is the concentration of the ligand.

RESULTS AND DISCUSSION

Fig. 1 shows a representative elution profile of L-tryptophan–BSA binding. This is a regular Hummel-Dreyer pattern with one positive peak followed by a negative peak. Here we used the external calibration method. The left part profile has one peak only, (the negative peak), which is used for the correlation of the peak area with the concentration of the L-tryptophan. Such a correlation is expressed in Fig. 2. Since our integrator was not used for the external calibration method, we do not have integrator data for the correlation. Fig. 2 presents the results of only two techniques. The standard deviation for each value of the area is not shown in order to maintain the clarity of the graph. The uncertainty is within 2%. For example, the numerical values for the first pair in Fig. 2 are 0.74 ± 0.01 (planimeter); 0.82 ± 0.02 (geometry), both in the unit of cm^2.

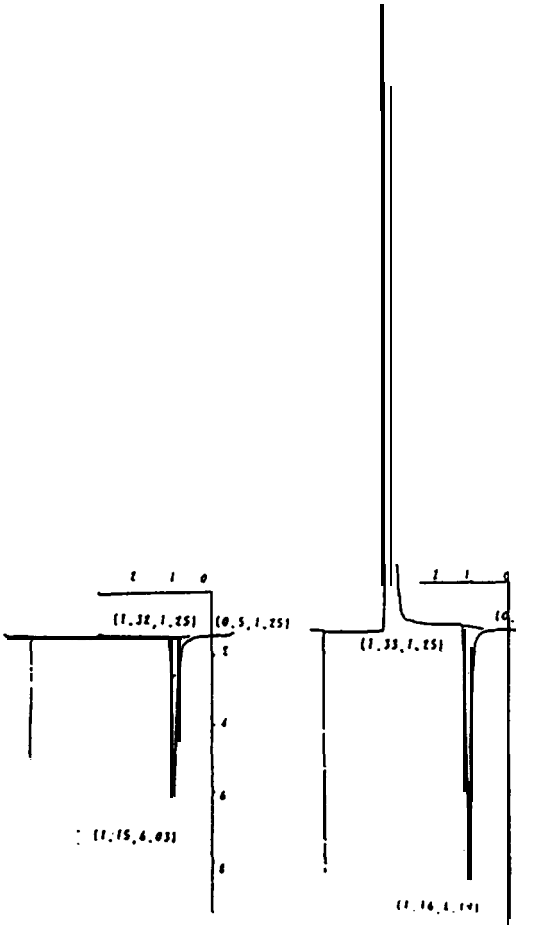

Fig. 1. Elution profile of the binding of L-tryptophan to BSA. Column: Protein Pack I-125 (Waters Assoc.), Detector: UV at 280 nm, 0.4 AUFS. Flow-rate: 1.0 ml/min. Mobile phase: 10.29 μM (see Table I). Sample: 100 μl of G and F (see Table II).

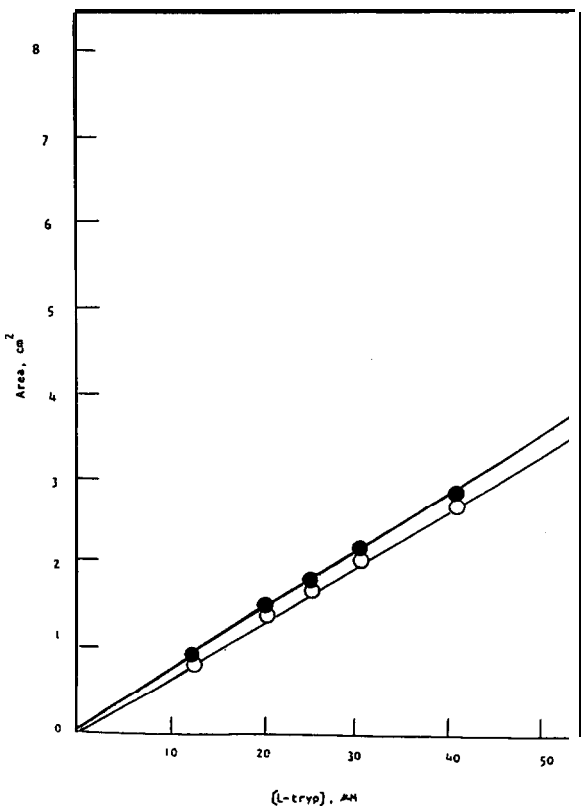

Fig. 2. Correlation of the area of the negative peak with the concentration of L-tryptophan for the external calibration method using planimeter and geometric calculation methods. ● = Geometric calculation; 0 = planimeter.

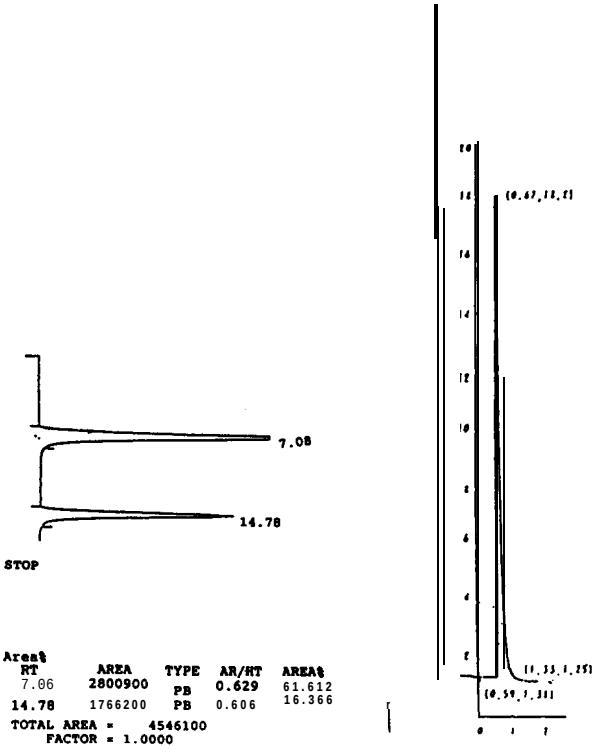

Fig. 3. Elution profile of the binding of L-tryptophan to BSA. Column: Protein Pack I-125 (Waters Assoc.), Detector: UV at 280 nm, 0.4 AUFS. Flow-rate: 100 μl of B (see Table II). Area measured by integrator and geometric calculation methods. RT= Retention time in min; AR = area; HT = height; PB = peak separation code.

The negative peak area of the right profile in Fig. 1 is converted to $[L]_b$ by reading the calibration curve (a straight line) in Fig. 2. The coordinates of the triangular vertices are indicated in both negative peaks, (one for the correlation and the other for the determination of $[L]_b$). These are used for geometric calculations. To use planimetry for the estimation of the area, no such coordinates are necessary. Although there is some discrepancy between geometry and planimeter readings as shown in the calibration curve (Fig. 2), such a discrepancy is rather negligible in view of the certain uncertainty involved in the experiment.

Fig. 3 shows a representative profile of L-tryptophan-BSA binding, using the internal calibration method. In order to emphasize the use of the integrator, we choose the two-positive-peak pattern as representative. For a two-positive-peak profile, we can use all the three techniques (planimeter, integrator and geometry) to evaluate the area of the second peak, and we did use all the three techniques. The left part in Fig. 3 was printed by the integrator with elution time and areas on the same paper; this is for illustration only. The right part pattern was obtained simultaneously with the left part pattern by the regular recorder which we used for planimeter and geometry readings. The coordinates are given to indicate the use of geometrical calculation. In the internal calibration method we do not need a correlation graph between the area and the concentration of L-tryptophan as in the external calibration. Instead we plot the area versus the amount of L-tryptophan in mol in five different mobile phases, each of which was

injected with five samples of different L-tryptophan concentrations, respectively. The extrapolation to zero abscissa gives the quantity L_b, the absolute amount of tryptophan bound to the protein. Such plots are shown in Figs. 4, 5, 6. They represent three different methods for the evaluation of areas: planimeter, integrator and geometry. Again, the uncertainty in each experiment point with respect to area is within 2%. The scatter is somewhat greater with the integrator than with the other two techniques.

In Tables III and IV we list the calculations of binding quantities $\bar{r}$ and $\bar{r}/[L]_f$, where [L], is the concentration of free L-tryptophan. Table III is based on external calibrations while Table IV is based on internal calibrations. We use the concentration of L-tryptophan in the mobile phase as the concentration of free L-tryptophan in the dynamic equilibrium. The Scatchard plot is shown in Fig. 7. The five straight lines represent the results of five different techniques. They do not superimpose into a single line, but the experimental points cluster reasonably together, indicating the feasibility in using any of them to study drug-protein binding phenomena. The integrator reading seems to show a relatively larger discrepancy from the other techniques. It is, however, the simplest, fastest and least labourious technique.

The binding parameters n and k are given in Table V. We see that the numerical values of

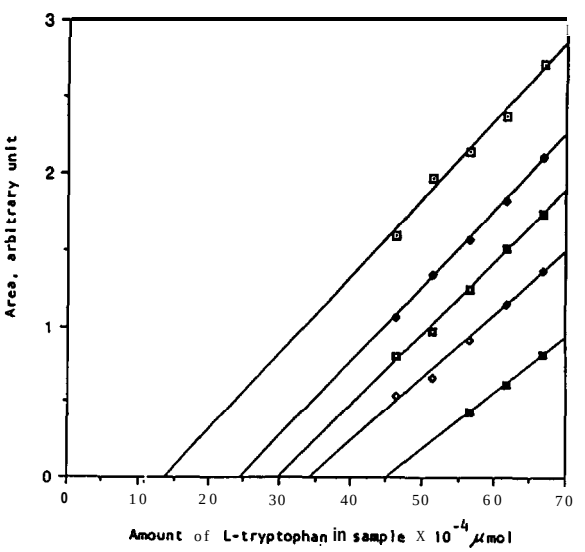

Fig. 4. Internal calibration for the determination of the amount of L-tryptophan that binds to BSA using the integrator method. Lines connect equal concentrations of L-tryptophan. From left to right: 10.29 μM, 20.58 μM, 25.73 μM, 30.87 μM, 41.16 μM.

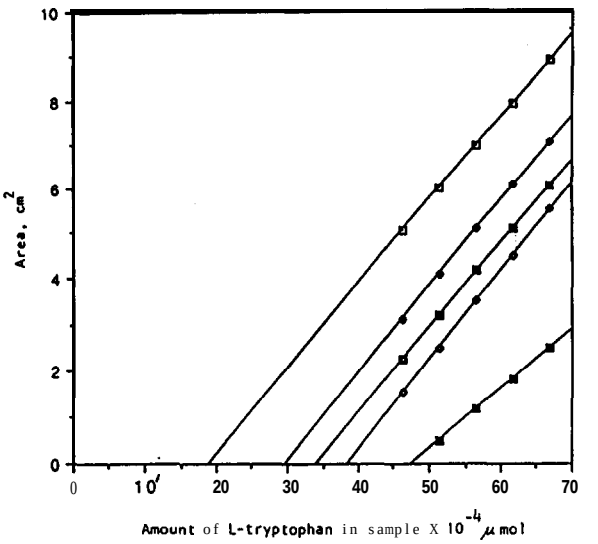

Fig. 5. Internal calibration for the determination of the amount of L-tryptophan that binds to BSA using the geometric calculation method. Concentrations of L-tryptophan as in Fig. 4.

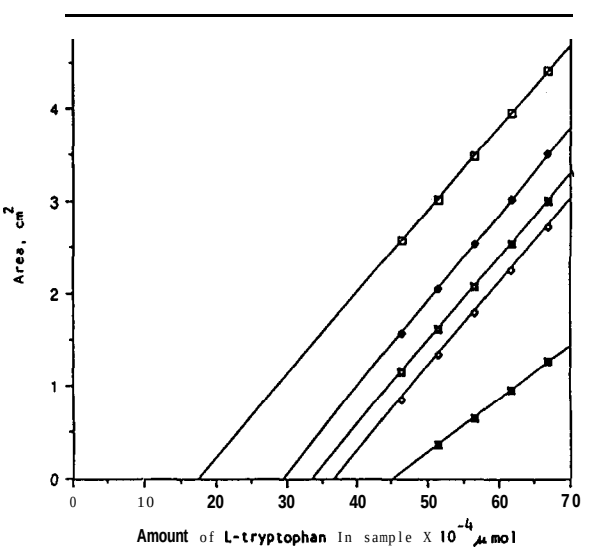

Fig. 6. Internal calibration for the determination of the amount of L-tryptophan that binds to BSA using the planimeter method. Concentrations of L-tryptophan as in Fig. 4.

TABLE III

L-TRYPTOPHAN-BSA BINDING BY EXTERNAL CALIBRATION ([p] = 66.09 μM; p = protein)

$[L]_f$ (μM)	$[L]_b$ (μM)	$\bar{r} = [L]_b/[p]$	$\bar{r}/[L]_f \times 10^4$
Planimeter			
10.3	17.5	0.265	2.60
20.6	28.9	0.437	2.10
25.7	33.0	0.499	1.90
30.9	38.1	0.576	1.90
41.2	45.4	0.687	1.70
Geometric calculation			
10.3	18.0	0.272	2.60
20.6	28.4	0.430	2.10
25.7	34.7	0.524	2.00
30.9	37.8	0.572	1.90
41.2	45.9	0.695	1.79

these two parameters are reasonably in agreement to each other. The n values are higher than those reported in the previous publication [S]. This is due to the fact that in ref. S the plot was not started from the origin. We believe that the

TABLE IV

L-TRYPTOPHAN-BSA BINDING BY INTERNAL CALIBRATION (p = 66.09. 10^{-4} μmol; p = amount of protein)

Mobile phase $[L]_f$ (μM)	Intercept L_b ($\times 10^{-4}$ μmol)	$\bar{r} = L_b/p$	$\bar{r}/[L]_f \times 10^4$
Integrator			
10.3	14.0	0.212	2.06
20.6	24.0	0.363	1.76
25.7	28.0	0.424	1.65
30.9	32.0	0.484	1.60
41.1	38.0	0.575	1.40
Geometric calculation			
10.3	19.1	0.289	2.81
20.6	29.8	0.451	2.19
25.7	34.2	0.517	2.01
30.9	38.2	0.583	1.89
41.2	46.1	0.698	1.69
Planimeter			
10.3	***17.4***	***0.263***	***2.55***
20.6	***29.4***	***0.445***	***2.16***
25.7	***33.6***	***0.509***	***1.98***
30.9	***37.0***	***0.559***	1.81
41.2	45.2	0.683	1.66

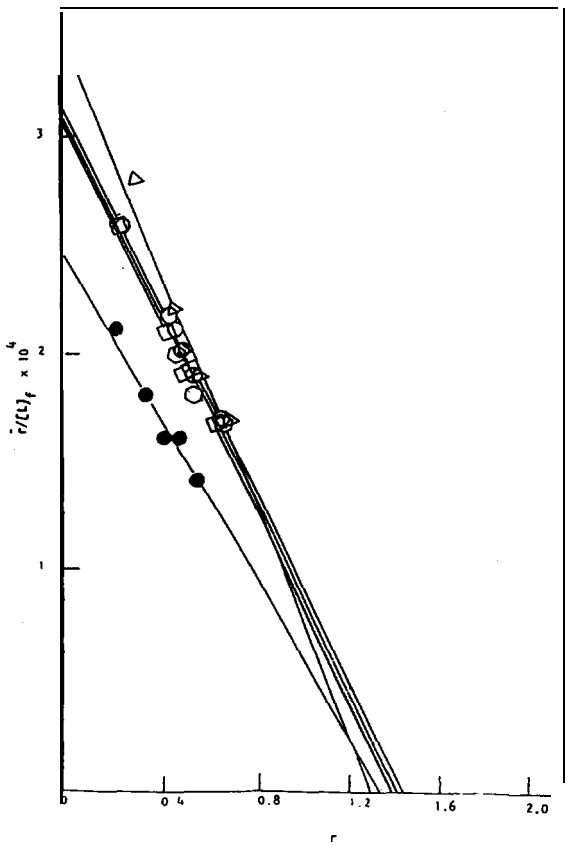

Fig. 7. Scatchard plot for the binding of L-tryptophan by BSA. ● = Internal by integrator; A = internal by geometric calculation; ⬡ = internal by planimeter; □ = external by planimeter; 0 = external by geometric calculation.

results reported in this paper are more reliable than all those previously published including those classical ones [9–11]. The reason is that we reached the conclusions with two different methods (i.e. external calibration and internal cali-

TABLE V

BINDING PARAMETERS OF L-TRYPTOPHAN-BSA SYSTEM

Area evaluation method	Internal (I) or external (E)	n	$k \times 10^4$
Planimeter	I	1.41	2.22
	E	1.44	2.22
Geometric calculation	I	1.29	2.72
	E	1.43	2.21
Integrator	I	1.35	1.80

bration) and three different techniques (planimeter, integrator and geometry). The numerical values as listed in Table V, while not identical, are in good agreement within the experimental error.

CONCLUSIONS

On the basis of our experience in the study of binding of L-tryptophan to serum albumin using the Hummel-Dreyer method, we conclude that any of the following techniques would reach almost the same conclusion: external calibration/planimeter reading, external calibration/geometric reading, internal calibration/planimeter reading, internal calibration/geometric reading and internal calibration/integrator reading.

The choice of any method depends on experimental conditions including laboratory facilities available. We were reminded by a referee that nowadays integrators are available that can handle negative peaks . If that is the case, we shall be able to use an integrator to evaluate the areas of the peaks obtained with external calibration method as well as internal calibration method of the Hummel-Dreyer elution pattern. This would further facilitate the study of drug-protein bindings.

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