Furosemide Binding by Human Albumin: Comparison of Two Methods of Fluorescence Quenching Analysis

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Abstract □ Disagreement exists over the primary-site binding constant for the interaction of furosemide with human albumin. Disagreement also exists over which experimental methods are accurate in this particular interaction. Therefore, furosemide binding by human albumin was examined using albumin fluorescence quenching by both the method of Levine and the method of Steiner *et al.* The binding constants obtained by each method differed greatly, with the results of the latter method being similar to those of other experimental methods. It was concluded that the method of Levine overestimates the binding constant for this drug–protein interaction.

Keyphrases □ Furosemide—binding by human albumin, comparison of two methods of fluorescence quenching analysis □ Binding—furosemide by human albumin, comparison of two methods of fluorescence quenching, analysis □ Human albumin—furosemide binding, comparison of two methods of fluorescence quenching analysis

The binding of a drug by blood proteins can influence the therapeutic, pharmacodynamic, and toxicological actions of the drug (1). Studies of drug binding by albumin are important since this is the major plasma protein responsible for the nonspecific binding of drugs (2). Most drugs that are extensively bound by plasma proteins are anions that bind to albumin (3), and these ligands can undergo competition for the limited number of albumin binding sites (4).

At low concentrations in plasma, furosemide is extensively bound exclusively or almost exclusively to albumin (5). However, there has been disagreement over the magnitude of the binding constant for the interaction of furosemide with its primary binding site on human albumin. Using the method of intrinsic protein fluorescence quenching and a new method of data analysis, a primary-site binding constant of $1.3 \times 10^7\ M^{-1}$ has been obtained for the interaction of furosemide with human albumin (6). As shown in Table I, this value considerably exceeds other reported values obtained using various experimental methods and conditions. It was suggested that the reason for the large affinity constant obtained using the new method of data analysis is that other experimental methods, such as equilibrium dialysis, give erroneously low binding constant values for strongly bound ligands (6). Previous work (13) has provided a theoretical alternative for this large binding constant obtained for the interaction of furosemide with human albumin; *i.e.*, the new method of data analysis employed overestimates the binding constant for certain interactants.

As discussed previously (13), the method of Levine is based on assumptions that are not strictly valid when the protein contains more than one binding site for the ligand. When this is the case, and with a low value of n_1k_1/n_2k_2, where n_i is the number of binding sites of class i and k_i is the site binding (affinity) constant for binding sites of class i, the method of Levine will, theoretically, overestimate the value of k_1 (13).

A third possible explanation for the large value of k_1 obtained using the new method of data analysis (6) is that different experimental conditions were employed. Factors such as the pH, temperature, and buffer system used, the method of albumin isolation, and presence of fatty acids can affect both the number and the affinity of binding sites (14).

Since the binding of a drug by albumin may greatly affect the *in vivo* actions of the drug, it is important that large discrepancies in reported binding constants be resolved. It is also important that possible limitations of new methods of data analysis be examined both theoretically and experimentally, since many investigators may eventually employ the new method. Theoretical limitations of the method of Levine were discussed previously (13). To evaluate experimentally the limitations of this method, the binding of furosemide by human albumin was re-examined using the quenching of intrinsic protein fluorescence by both the Levine (6) and Steiner *et al.* (15) methods. The method of Steiner *et al.* is theoretically sound and applicable when the protein contains more than one binding site for the ligand.

EXPERIMENTAL

Materials and Methods—Fatty acid free human albumin[1] and furosemide[2] as a pure powder were used as received. All protein solutions were prepared using 0.125 *M* phosphate buffer at pH 7.4. The buffer was prepared immediately prior to the experiment using analytical grade monosodium and disodium phosphates and water purified by reverse osmosis followed by distillation.

Fluorescence measurements were made with a spectrofluorometer[3], and spectrophotometric measurements were made with a spectrophotometer[4]. Rectangular quartz fluorescence cells with a path length of 10 mm were used for both fluorescence and spectrophotometric measurements.

Albumin solutions of 1.9, 6.0, 10, 50, and 100 μmoles/liter were utilized in this study. All albumin solutions were corrected for moisture content and purity and were ~2-hr old at the start of each experiment. Furosemide solutions (0.3–1.2 mmoles/liter) were prepared immediately prior to use and were protected from light. Furosemide solutions were prepared using a portion of the albumin solutions under investigation to avoid dilution of the albumin solution upon addition of drug.

The decrease in the magnitude of intrinsic albumin fluorescence upon the addition of microliter volumes of furosemide solution to 2 ml of protein solution was measured at ambient temperature (24.5 ± 0.8°). Excitation and emission wavelengths of 292 and 370 nm, respectively, were used to minimize the absorption of light by furosemide. Excitation and emission slit widths of 2 and 12 nm, respectively, were used, except at the higher protein concentration (100 μmoles/liter) where an emission slit width of 10 nm was utilized. The fluorescence intensity of cells containing buffer only and albumin only, at the same concentration as the sample cell, was measured prior to each measurement of sample cell fluorescence intensity. These reference solutions were used to correct the

[1] Miles Laboratories, Inc., Elkhart, IN 46514.
[2] Hoechst-Roussel Pharmaceuticals, Inc., Somerville, NJ 08876.
[3] Model 650-10M, Perkin-Elmer Corp., Norwalk, CT 06856.
[4] DU-8, Beckman Instruments, Inc., Irvine, CA 92713.

0022-3549/83/0500-0542$01.00/0

Table I—Binding Constants for the Interaction of Furosemide with Human Albumin Previously Reported Using a Variety of Experimental Methods and Conditions

n_1	k_1, kl/mole	n_2	k_2, kl/mole	Reference
1	13,000	—	—	(6)
1	27	—	—	(7)
1.42	50.7	3.4	15.8	(8)
—	237[a]	—	31[a]	(9)
0.90	168	4.56	9.6	(10)
1.3	26.8	2.2	0.59	(11)
1	120	3	3	(12)

[a] Rough approximation.

sample fluorescence for background fluorescence and any variations in instrument response or protein fluorescence. The solutions were only exposed to the excitation light for the few seconds required for each measurement.

Four fluorescence quench titrations were performed at each albumin concentration investigated except 100 μmoles/liter, where six titrations were conducted. For a given albumin concentration, the fluorescence quench titrations were identical in that fluorescence measurements were always made at the same total molar drug concentration to total molar protein concentration ratio, D/P. Each sample fluorescence measurement was corrected for the inner filter effect based on the absorbance of the solution at the wavelengths of excitation and emission (16). Three absorbance titrations were performed at each albumin concentration investigated. The absorbance titrations at any given protein concentration were identical to the fluorescence quench titrations at the same protein concentration, in that fluorescence and absorbance measurements were obtained at the same values of D/P. The mean absorbance of the sample solutions at both 292 and 370 nm at each D/P value was used to correct the corresponding fluorescence measurements at the same protein concentration and D/P value.

After correcting for the inner filter effect, each fluorescence measurement was converted to percent initial fluorescence. The mean percent initial fluorescence at each value of D/P at each protein concentration was calculated and plotted as a function of D/P. These quench curves were used to calculate values of r, the ratio of molar concentration of bound ligand to total molar protein concentration, and $[L]$, the molar concentration of free ligand at each protein concentration using both the method of Levine (6) and the method of Steiner *et al.* (15). The values of r and $[L]$ were then used to construct a Scatchard plot (17) based on the following equation:

$$r = \sum_{i=1}^{m} \frac{n_i k_i [L]}{1 + k_i [L]} \qquad \text{(Eq. 1)}$$

Binding constants were obtained by a computer fit (18) of the binding data obtained using the method of Steiner *et al.* (15). Since the method of Levine (6) assumes that essentially no ligand is bound to secondary albumin binding sites until the primary binding site is saturated, the data obtained by this method are treated on the assumption that albumin contains a single binding site for ligand. In this case, a Scatchard plot (17) and linear regression can be used to obtain values of n_1 and k_1 or Eq. 2 (6) can be used to calculate k_1 directly from the quench curve:

$$k_1 = \frac{Q}{(1 - Q)P[(D/P) - Q]} \qquad \text{(Eq. 2)}$$

where: $Q = (F_0 - F)/m$, F_0 = fluorescence at $D/P = 0$, F = fluorescence at a given value of D/P, m = slope of the initial portion of the quench curve, *i.e.*, at low values of D/P, and P = total molar albumin concentration.

Using Eq. 2, a value of k_1 can be calculated for each experimental point on the quench curve that lies above the straight line fitted to the initial portion of the quench curve. Equation 2 assumes that $n_1 = 1$ and that $Q = r$.

RESULTS

The quench curves obtained at each albumin concentration investigated are presented in Fig. 1. For clarity, a few experimental points obtained at the lower albumin concentrations and D/P values >7, and at the higher albumin concentrations and D/P values <1, are not presented in this figure. As expected, the mean percent initial fluorescence at any given D/P value decreases as the albumin concentration increases. The coefficient of variation for the individual values of mean percent initial

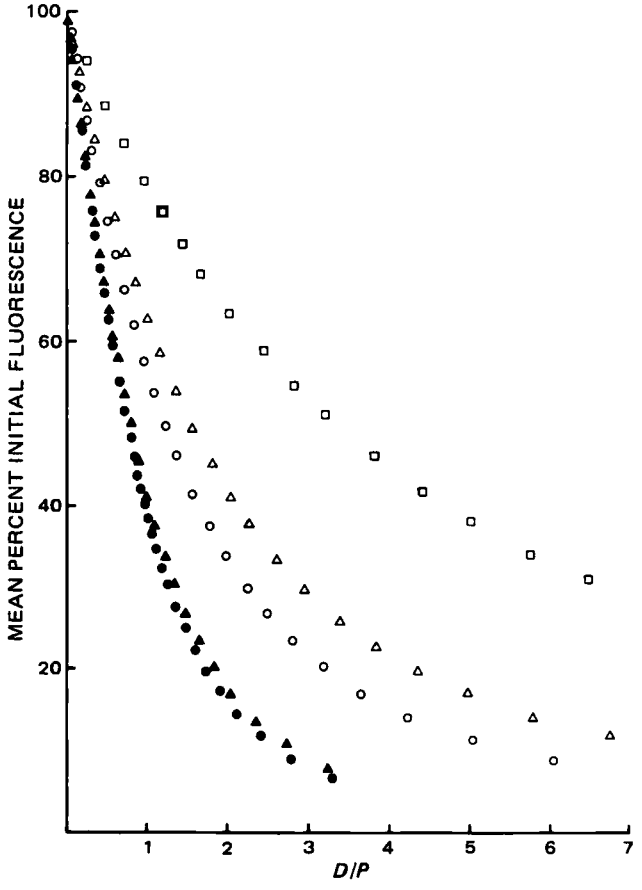

Figure 1—*Fluorescence quench curves obtained for the interaction of furosemide with 1.9 (□), 6.0 (△), 10 (○), 50 (▲), and 100 (●) μmoles/liter of human albumin.*

fluorescence ranged from 0.001 to 0.043 and the standard deviation for these individual values ranged from 0.1 to 0.9 for all but the largest albumin concentration. At an albumin concentration of 100 μmoles/liter, the coefficient of variation for the individual values of mean percent initial fluorescence ranged from 0.001 to 0.104 and the standard deviation for these individual values ranged from 0.1 to 2.5. As a result of the larger data variability observed at an albumin concentration of 100 μmoles/liter, six, rather than four, fluorescence quench titrations were performed at this protein concentration. The larger variability observed at the highest albumin concentration may be due to the larger inner filter effect and/or the smaller decrease in observed fluorescence between additions of furosemide solution. Data could not be obtained at higher protein concentrations due to the large sample absorbance encountered at relatively low D/P values.

Figure 1 shows that the quench curves obtained at the two largest albumin concentrations investigated (50 and 100 μmoles/liter) are identical at low D/P values and are very similar throughout the D/P range investigated. The portion of these curves that is identical represents the percent of initial fluorescence observed when all of the added ligand is bound (19), *i.e.*, a plot of percent initial fluorescence as a function of r. The fact that these two quench curves are very similar at higher D/P values suggests that a larger portion of the quench curve obtained at an albumin concentration of 100 μmoles/liter represents a plot of percent initial fluorescence as a function of r. Therefore, the quench curve obtained at an albumin concentration of 100 μmoles/liter and the method of Steiner *et al.* (15) were used to construct a Scatchard plot (17) of the data obtained at albumin concentrations of 1.9, 6.0, and 10 μmoles/liter. The quench curve obtained at an albumin concentration of 50 μmoles/liter could not be used due to its similarity to the quench curve obtained at an albumin concentration of 100 μmoles/liter.

The Scatchard plot obtained using the method of Steiner *et al.* (15) is presented in Fig. 2. This plot becomes unusual in appearance at values of $r > \sim 1.5$, in that the data obtained at different protein concentrations no longer represent a single curve, and the instantaneous slope of each curve becomes increasingly negative. This shows that the quench curve obtained at an albumin concentration of 100 μmoles/liter does not rep-

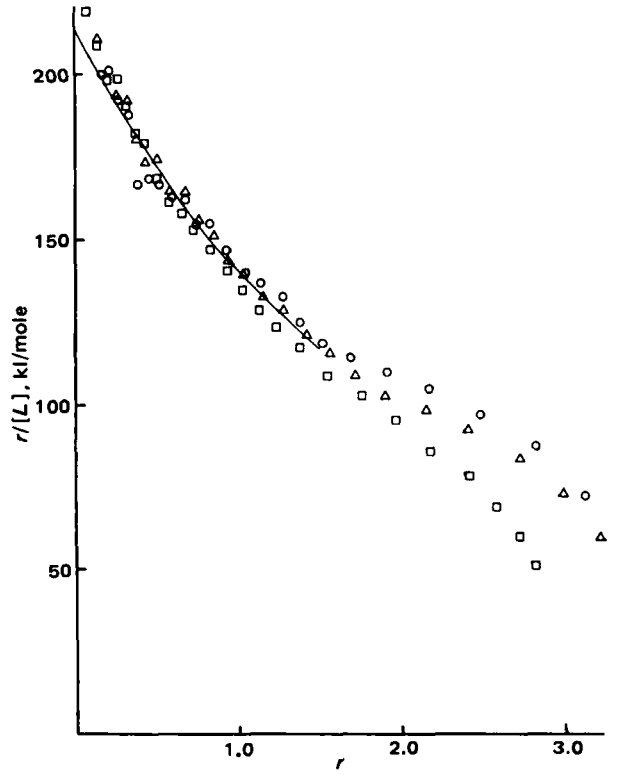

Figure 2—*Scatchard plot obtained for the binding of furosemide by 1.9 (□), 6.0 (△), and 10 (○) μmoles/liter of human albumin using the method of Steiner* et al. *(15).*

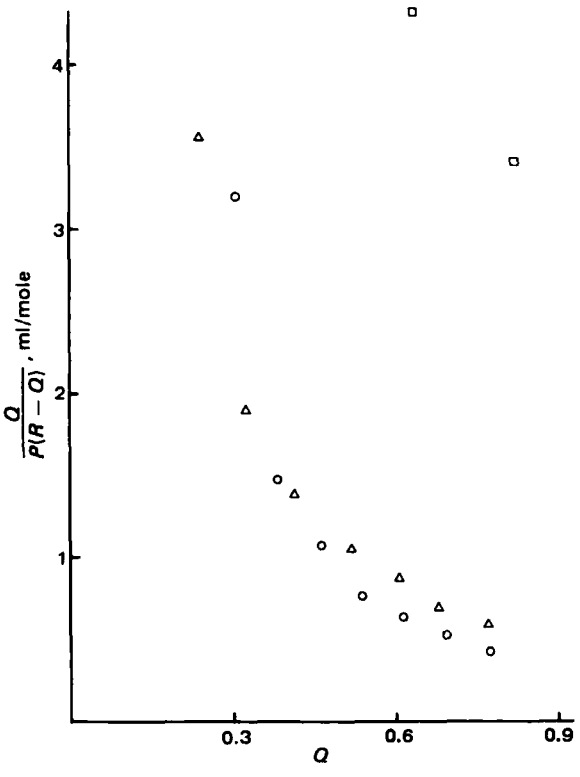

Figure 3—*Scatchard plot obtained for the binding of furosemide by 1.9 (□), 6.0 (△),and 10 (○) μmoles/liter of human albumin using the method of Levine (6).*

resent the stoichiometric binding of ligand at D/P values >1.5. Use of this quench curve at these values results in an overestimate of the affinity of albumin for ligand at higher r values and this results in an increase in the slope of the Scatchard plot at r >1.5. Assuming two classes of binding sites, a fit of the data of r <1.5 presented in Fig. 2 to Eq. 1 yields $n_1 = 0.70$, $k_1 = 166$ kl/mole, $n_2 = 5.5$, and $k_2 = 17.7$ kl/mole. These constants were used to compute the solid line in Fig. 2. The deviation of this line at lower r values results from the computer fit of the data to Eq. 1, rather than to $r/[L]$ as a function of r.

The method of Levine (6) also was used to interpret the quench curves obtained at albumin concentrations of 1.9, 6.0, and 10 μmoles/liter. Using Eq. 2, a value of k_1 was calculated for each experimental point on the quench curve of D/P <1 that lies above the straight line fitted to the initial portion of the quench curve. The results are presented in Table II.

The method of Levine (6) was also used to construct a Scatchard plot (17) of the data obtained at albumin concentrations of 1.9, 6.0, and 10 μmoles/liter. This Scatchard plot is presented in Fig. 3. As discussed, linear regression analysis of the Scatchard plot (17) is used in the method of Levine (6) to obtain values of n_1 and k_1. Values of n_1 and k_1 were not obtained for the data of Fig. 3 due to the nonlinearity of this plot.

Table II—Affinity Constant (k_1) Values Obtained for the Binding of Furosemide by its Primary Site on Human Albumin Using the Method of Levine (6) and Eq. 2

1.9 μmole/liter[a] (m = 25.2)		6.0 μmole/liter[a] (m = 48.4)		10 μmole/liter[a] (m = 55.1)	
D/P	k_1, Ml/mole	D/P	k_1, Ml/mole	D/P	k_1, Ml/mole
0.47	18.8	0.25	4.68	0.31	4.60
0.71	11.8	0.35	2.81	0.40	2.37
0.95	19.0	0.47	2.40	0.51	2.00
		0.60	2.18	0.61	1.64
		0.72	2.21	0.71	1.66
		0.84	2.16	0.82	1.69
		0.99	2.56	0.95	1.86
Mean	16.5		2.72		2.26
SD	4.08		0.898		1.06
CV	0.247		0.330		0.469

[a] Albumin concentration.

DISCUSSION

The quenching of the intrinsic fluorescence of human albumin upon the binding of furosemide was used to study this drug–protein interaction. The quench curves obtained (Fig. 1) show that at the lower albumin concentrations examined, only a few, if any, of the experimental points lie on the quench curve obtained at the largest albumin concentration. This shows that only a small section, if any, of the initial portion of the quench curves obtained at the lowest three albumin concentrations represents the fluorescence observed when all of the added furosemide is bound. Thus, the initial apparently linear portion of the quench curves obtained at albumin concentrations of 1.9, 6.0, and 10 μmoles/liter do not represent the stoichiometric binding of furosemide as is assumed by the method of Levine.

Binding constants for both the primary and secondary binding sites on human albumin for furosemide were determined from the quench curves using the method of Steiner *et al.* (15). The quench curve obtained at an albumin concentration of 100 μmoles/liter through a D/P value of 1.5 was used as the stoichiometric curve. If any portion of this curve does not represent the stoichiometric binding of furosemide, the binding constants obtained will be too high rather than too low. The values of the binding constants obtained in this study using the method of Steiner *et al.* (15) are similar to the values obtained previously (Table I) with the exception of the k_1 value obtained using the method of Levine (6). The lower value of n_1 and the higher value of n_2 obtained in the present study is at least partially due to the limited range of r values that could be obtained.

The quench curves obtained at albumin concentrations of 1.9, 6.0, and 10 μmoles/liter (Fig. 1) were also analyzed by the method of Levine (6) using both Eq. 2 and a Scatchard plot (17) (Fig. 3). The quench curves obtained at albumin concentrations of 50 and 100 μmoles/liter were not analyzed by this method, since they represent the stoichiometric binding of furosemide. The values of k_1 obtained using Eq. 2 are presented in Table II. For each albumin concentration examined, the k_1 value obtained appears to be dependent on the experimental point (D/P) used in the calculation of k_1. As the D/P value increases, the value of k_1 appears to decrease to some minimum value and then increase. The value of k_1 does not vary in the random fashion expected due to experimental error. This apparent dependence of k_1 on D/P implies that one or more of the assumptions of this method of data analysis is invalid for this particular drug–protein interaction. The coefficient of variation values for the mean

values of k_1 are very high relative to the coefficient of variation values obtained for the individual experimental points of the quench curve. The nonlinearity of the Scatchard plot obtained using this method of data analysis (Fig. 3) also implies that one or more of the assumptions of this method is invalid for this particular drug–protein interaction.

The Scatchard plot previously obtained by this method for the binding of furosemide by human albumin was not published (6). Thus, the experimental data suggest that one or more of the assumptions on which the method of Levine is based is invalid as was previously suggested (13) based on theoretical considerations.

The mean value of k_1 ($1.65 \times 10^7\ M^{-1}$) obtained using Eq. 2 and an albumin concentration of 1.9 μmoles/liter is very similar to the value of $1.3 \times 10^7\ M^{-1}$ previously obtained (6) using the same albumin concentration, experimental conditions, and method of data analysis. As shown in Table II, the mean value of k_1 decreased and the value of m increased as the albumin concentration increased. This is to be expected, since the initial apparently linear portion of the quench curve obtained at each protein concentration does not represent the stoichiometric binding of the ligand. This shows how difficult it is to determine the stoichiometric region of a quench curve when data are collected at a single protein concentration. The method of Levine assumes that this stoichiometric region can be determined using a single protein concentration.

Based on these results, the reason for the large value of k_1 previously obtained (6) for the binding of furosemide by human albumin using the new method of data analysis is not that other experimental methods, such as equilibrium dialysis, resulted in erroneously low values of this constant. Since the same set of data was analyzed by two different methods in the present investigation, the large difference in the values of k_1 obtained cannot be attributed to a difference in experimental conditions. Instead, the results of this study show that the new method of data analysis (6) overestimates the value of k_1 in this particular drug–protein interaction. As discussed previously (13), the method of Levine will, theoretically, overestimate the value of k_1 when the protein contains more than one binding site and has a low value of n_1k_1/n_2k_2. The experimental results of the present work support these theoretical findings. This overestimation of k_1 also results from the inaccurate determination of the stoichiometric region of the fluorescence quench curve obtained at a single albumin concentration.

As discussed previously (13), the method of Levine (6) has several advantages over the method of Steiner *et al.* (15) and results in accurate estimates of k_1 for certain ligand–protein interactions, such as the binding of bilirubin by human albumin. However, the method of Levine will overestimate the value of k_1 when the protein contains more than one binding site for the ligand and the value of n_1k_1/n_2k_2 for the interaction is low. The binding of furosemide by human albumin is an example of such an interaction. For this reason, the binding constant obtained using this new method of data analysis (6) should always be verified by an additional experimental method. When a drug–protein interaction is being investigated for the first time, and the method of intrinsic protein fluorescence quenching is the only experimental method used, the data should always be analyzed by the method of Steiner *et al.* (15).

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ACKNOWLEDGMENTS

The author wishes to thank Fredrich Harding and Charles Steiner for their technical assistance in the collection of preliminary data. The author also wishes to thank Hoechst-Roussel Pharmaceuticals, Inc. for the gift of furosemide.