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Measurement of Protein Binding by Ultracentrifugation

YASUO MATSUSHITA* and IKUO MORIGUCHI

School of Pharmaceutical Sciences, Kitasato University, Shirokane, Minato-ku, Tokyo 108, Japan

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Conditions and the limits of measurement for protein-binding studies by the ultracentrifugation (UC) method were investigated, and the UC method was compared with the currently used equilibrium dialysis (ED) method in terms of accuracy and reliability. Serum albumin (SA) concentrations of around 5.0×10^{-5} M and drugs with molecular weights of less than 400 were suitable for the UC method. The optimal rotation speed was considered to be 30000 rpm ($61380 \times g$). The UC method and ED method were compared in measurements of the binding of 4′-hydroxyazobenzene-2-carboxylate, 2-naphthoate, salicylate and warfarin to bovine SA and human SA. The UC method was confirmed to be comparable with the ED method in terms of reliability and rather superior in terms of reproducibility, especially at low drug concentrations.

Keywords——protein binding; bovine serum albumin; human serum albumin; equilibrium dialysis method; ultracentrifugation method

Studies on the binding of drugs to serum albumin (SA) are important in relation to drug efficacy and clinical application of drugs, and much work has been done to elucidate the binding mechanisms. Conventionally, binding of drugs to SA has been measured by several methods. Among them, the equilibrium dialysis (ED) method, a classical one, has been employed extensively as a direct method to measure the binding.[1,2] Since the ED method requires no special devices or equipment, it has been used widely.[3] However, its reproducibility is problematic. The main drawback of the ED method is the adsorption of drugs on the dialysis membrane. It has been reported[4] that, at low concentration, as much as 20% of a drug may be adsorbed on the dialysis membrane and such adsorption may be variable from bag to bag. Recently, application of dialysis cells has greatly improved reproducibility. Nonetheless, problems still remain in measurements at low drug concentrations. Drugs with higher activity have been developed in recent years, and very low concentrations of unbound drugs in plasma may be sufficient for drug action. Therefore, accurate determination of drug binding at low concentrations of drugs has become very important. In view of these facts, we chose to investigate the ultracentrifugation (UC) method among available methods other than the ED method because of its high degree of reproducibility at low drug concentrations.

It has been reported that some discrepancies are seen between the results obtained by the UC method and those by the bag-type ED method.[5a] In the present study, the effects of SA concentration, molecular weight of drugs, and conditions of measurements such as temperature and speed of rotation were investigated in order to clarify the reliability and limits of the UC method. Moreover, the bindings of SA with dyes and acidic drugs such as 4′-hydroxyazobenzene-2-carboxylate, 2-naphthoate, salicylate and warfarin was measured by the UC method and the cell-type ED method, and the results were compared.

Experimental

Materials——Bovine serum albumin (BSA, Fraction V) was purchased from Armour Pharmaceutical Co.,

Kankakee, and human serum albumin (HSA, Fraction V) was purchased from Miles Laboratories Inc., Elkhart. The molecular weight was assumed to be 67000,[6] and the concentration was determined by measuring the absorbance at 280 nm with $E^{1\%}_{1\,cm}=6.67$[6] and $E^{1\%}_{1\,cm}=5.30$[7] for BSA and HSA, respectively. 4′-Hydroxyazobenzene-2-carboxylate (HABCA, Daiichi Pure Chemicals Co., Ltd., Tokyo), 2-naphthoate (Tokyo Kasei Co., Tokyo), and salicylate (Wako Pure Chemical Industries, Ltd., Osaka) were used without further purification. Warfarin was generously provided by Eisai Co., Tokyo. All other reagents used were commercially available and of special grade. All final solutions were made with 0.15 M Tris–HCl buffer, pH 7.0.

Equilibrium Dialysis (ED) Method——Using 3-ml dialysis cells as devised by Goto *et al.*,[3] sample solutions were shaken for 16 h at 15 °C in a thermostat. In the dialysis, Visking dialysis membrane (36/32 type, Union Carbide Co.) was used after being boiled four times in distilled water. The decrease in the unbound drug content was measured, and from that the binding parameters were calculated. Each point in a Scatchard plot (Figs. 5—8) represents the mean value of four or five experiments. Adsorption of drugs on the membrane was negligible.

Ultracentrifugation (UC) Method——Solutions containing SA and drug, drug alone and SA alone at given concentrations were prepared so as to make a total volume of 6.0 ml in each case, and were centrifuged in a Hitachi 65P preparative ultracentrifuge using a 65TA rotor. The concentrations of unbound drug and SA remaining in the top fraction were measured. The centrifugation time was 15 h.[5a,b] After that, a 1.5 ml aliquot was sampled from each top fraction and the optical absorbance was measured. The amount of bound drug was determined as follows:

(amount of bound drug)
= {(absorbance of drug in the top fraction of drug alone solution)
−(absorbance of the top fraction of drug and SA solution)
+(absorbance of the top fraction of SA alone solution)}/
(molar absorptivity of drug).

The amounts of unbound drug and SA were spectrophotometrically measued with a Hitachi 200-20 type spectrophotometer. The measuring wavelengths for HABCA, 2-naphthoate, salicylate, and warfarin were 348, 231, 296, and 308 nm, respectively. Each point of a Scatchard plot (Figs. 4—8) represents the mean value of two or three experiments.

Viscosity——Viscosity measurements were carried out in an Ubbelohde type viscometer ($K=0.00407$, Shibata Chemical App. Mfg. Co., Ltd., Tokyo) at 15 °C. The flow time for water was 279.4 s. Density measurements were carried out using a pycnometer at 15 °C.

Calculation——Data obtained by the UC method and ED method were plotted according to Scatchard.[8] Since a curve (not a straight line) was generated by this plotting, calculation was done by using Karush's formula[9] with two kinds of binding sites or the stepwise model[10,11] described by Klotz.[10]

Karush's formula is given by Eq. 1,

$$r=n_1k_1C/(1+k_1C)+n_2k_2C/(1+k_2C) \tag{1}$$

where n_1 and n_2 are the numbers of primary and secondary binding sites, respectively, and k_1 and k_2 are the corresponding association constants. C is the free drug concentration and r is the amount (mol) of bound drug per mol of albumin. Parameters for the stepwise model, $PD_{i-1}+D \rightleftarrows PD_i$ ($i=1, 2, \cdots, N$), were calculated by using the values of n_1, n_2, k_1, and k_2 in Eq. 1 as follows:

$$K_i=(PD_i)/(PD_{i-1})(D)$$

$$K_1=k'_1+k'_2+\cdots\cdots\cdots+k'_N$$

$$K_1K_2=k'_1k'_2+k'_1k'_3\cdots+k'_{N-1}k'_N$$

$$\vdots \qquad \vdots \qquad \vdots$$

$$K_1K_2\cdots K_N=k'_1k'_2\cdots\cdots\cdots\cdots k'_{N-1}k'_N$$

where

$$N=n_1+n_2, \qquad k'_1=k'_2=\cdots=k'_{n_1}=k_1,$$

and

$$k'_{n_1+1}=k'_{n_1+2}=\cdots=k'_N=k_2$$

$$K_i=K_1\cdots K_i/K_1\cdots K_{i-1}$$

$$r=(K_1C+2K_1K_2C^2+\cdots+NK_1K_2\cdots K_NC^N)/$$
$$(1+K_1C+K_1K_2C^2+\cdots+K_1K_2\cdots K_NC^N)$$

For calculation of the stoichiometric equilibrium constant (K_i) by the stepwise model method, n_1 and n_2 obtained from Karush's equation were fixed at the nearest integer values and n_1, n_2, k_1 and k_2 were calculated again using the correlation coefficient between the observed and calculated values of r as an index. Then, k_1 in n_1 number and k_2 in n_2 number were aligned and numbered in one consecutive series. Various products of K were calculated, from which K_i was determined. Actually, K_i in the number of $n_1+n_2=N$ can be obtained. However, when plotting r against C, a curve was generated in which r tended to a particular value (n) with increase in C. This n was determined for each drug and then n number of K_i values were calculated. The curve fitting calculation was performed by means of a JEOL digital computer, model JEC-7E.

Results and Discussion

Influence of Albumin Concentration, Molecular Weight of Drugs, Rotation Speed, and Temperature on the Ultracentrifugation (UC) Method

The content of albumin remaining in the top fraction after centrifugation was measured at rotation speeds ranging from 26000 to 34000 rpm (46100—78800 $\times g$) and SA concentrations from 10^{-5} to 10^{-4} M (Fig. 1). At a speed of 26000 rpm (46100 $\times g$), the top fraction showed high absorbance at 280 nm, with 3.5 to 19% albumin (concentration ratio) remaining. However, with an increase in speed, lower absorbance was obtained at SA concentrations up to about 5.0×10^{-5} M, the ratio of albumin remaining in the top fraction being 1.5 to 3.5%. At an SA concentration of 5.0×10^{-5} M, a remaining ratio of approximately 1.5% was obtained at speed of 30000 (61380 $\times g$) and 32000 (69840 $\times g$) rpm. At higher SA concentrations, although the ratio of BSA (concentration ratio) remaining in the top fraction decreased, the amount of top fraction BSA as well as HSA became greater, accompanied by an increase in absorbance at 280 nm. This might lower the reliability of measurements of free drug concentration in the top fraction. Therefore, 30000 rpm centrifugation speed and approximately 5.0×10^{-5} M SA concentration were selected as standard conditions.

Next, in order to determine the effect of molecular size of drugs on sedimentation, a total of 15 drugs ranging in molecular weight from about 120 to 800 were checked for

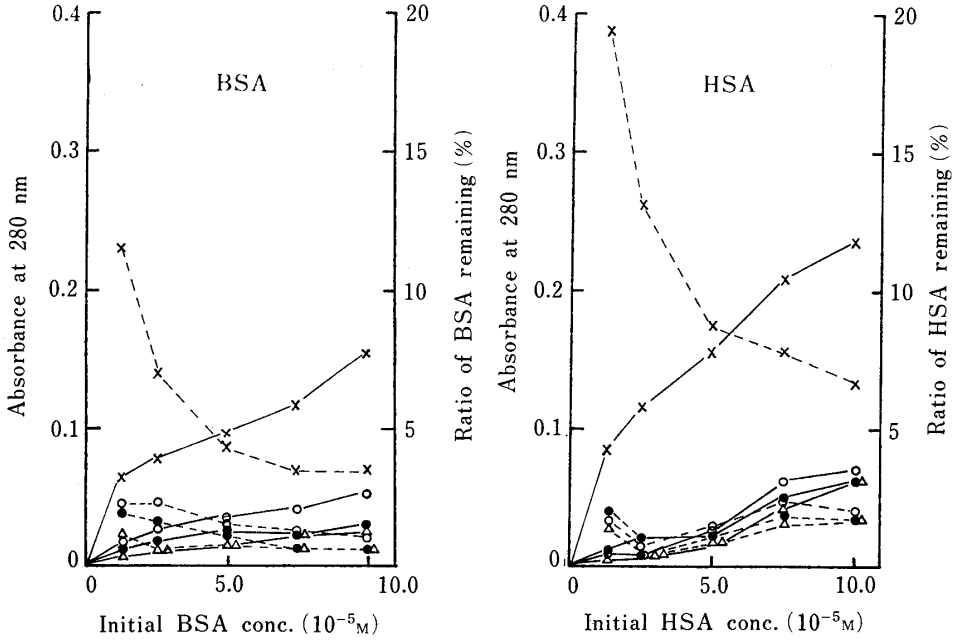

Fig. 1. Remaining BSA and HSA in the Top Fraction after Ultracentrifugation at Various Rotation Speeds for 15 h at 15 °C

Abscissa: initial concentration of SA (10^{-5}—10^{-4} M). Ordinates: absorbance at 280 nm (——) and the ratio of SA remaining (----) in the top fraction. Centrifugation speed (rpm): 26000 (×), 30000 (○), 32000 (●), and 34000 (△).

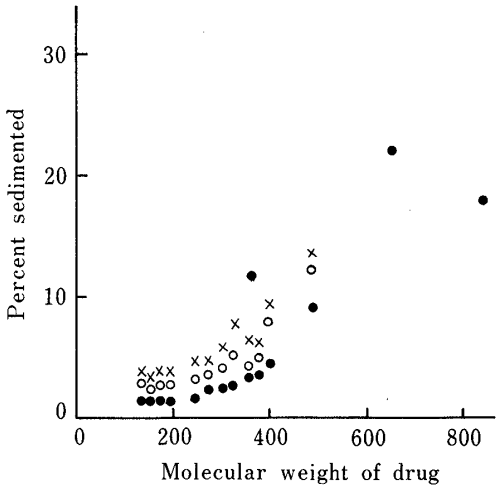

Fig. 2. Effect of Molecular Weight of Drugs on Sedimentation by Ultracentrifugation for 15 h at 15 °C

Sedimentation was measured at a concentration of 5.0×10^{-5} M and at three different speeds (● 30000, ○ 32000 and × 34000 rpm).

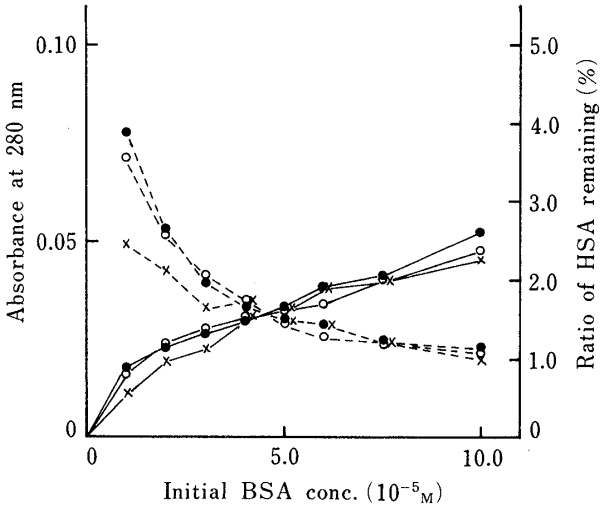

Fig. 3. Effect of Temperature on the Sedimentation of BSA by Ultracentrifugation

Absorbance at 280 nm (——) and the ratio of BSA remaining (– – – –) in the top fraction were determined after ultracentrifugation for 15 h at 30000 rpm at 15 °C (●), 23 °C (○) and 30 °C (×).

sedimentation at an SA concentration of 5.0×10^{-5} M and at three different rotation speeds, based on the above results. These 15 drugs included benzoate, *p*-hydroxybenzoate, HABCA, warfarin, methylene blue, indomethacin, sunset yellow, indigo carmine, congo red, erythrosine *etc.* At 30000 rpm, the sedimentation ratio remained 0.5—1.5% for drugs with molecular weights up to 300 (Fig. 2). Moreover, a small sedimentation ratio of 1.5—3% could be obtained even for drugs with molecular weights of 300 to 400. However, for drugs or dyes with higher molecular weight, the sedimentation ratio increased markedly. Even for low-molecular-weight substances, the sedimentation rate became somewhat higher at speeds of 32000 and 34000 rpm. Methylene blue and congo red, which gave abnormally high sedimentation rates for their molecular weights at a speed of 30000 rpm, were considered to form dimers. From these results, the optimal speed that minimizes the remaining SA in the top fraction and the sedimentation of drugs after centrifugation is considered to be 30000 rpm. It is likely that, at this speed, a reasonably accurate result can be obtained when the binding parameter is calculated with correction for SA remaining in the top fraction and drug sedimented by using reference solutions of SA alone and drug alone centrifuged along with the sample solutions.

Using BSA (10^{-5}—10^{-4} M), the ratio of albumin remaining in the top fraction after centrifugation for 15 h at 30000 rpm was determined at three different temperatures, 15, 23, and 30 °C (Fig. 3). As shown in Fig. 3, the pattern of albumin remaining in the top fraction showed no dependency on the temperature of centrifugation. Thus, temperature (within the above experimental range) did not affect the sedimentation of albumin.

Then, the relative viscosity (η_{rel}) of solutions of albumin and a mixture of albumin and drug was determined with an Ubbelohde viscometer at 15 °C using BSA and HSA at a concentration of 5.0×10^{-5} M. The η_{rel} for both the BSA and HSA solutions was 1.016. On the other hand, η_{rel} values for solutions of SA to which 10^{-5} and 10^{-3} M warfarin or HABCA had been added ranged from 1.017 to 1.022, showing little or no difference from the value obtained for SA alone. Thus, the influence of viscosity change on the centrifugation can be neglected.

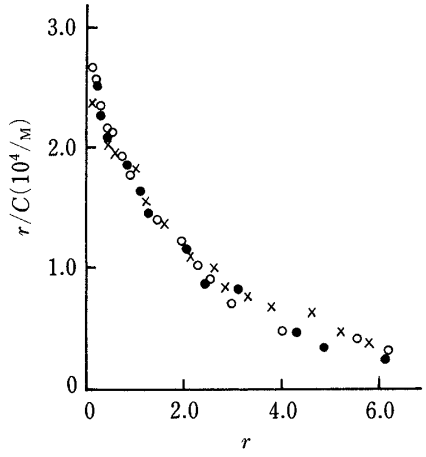

Fig. 4. Scatchard Plots for the Binding of HABCA to Various Concentrations of BSA Determined by the UC Method at 15 °C

BSA concentration: 0.833×10^{-5} M (●), 2.08×10^{-5} M (○), 5.0×10^{-5} M (×).

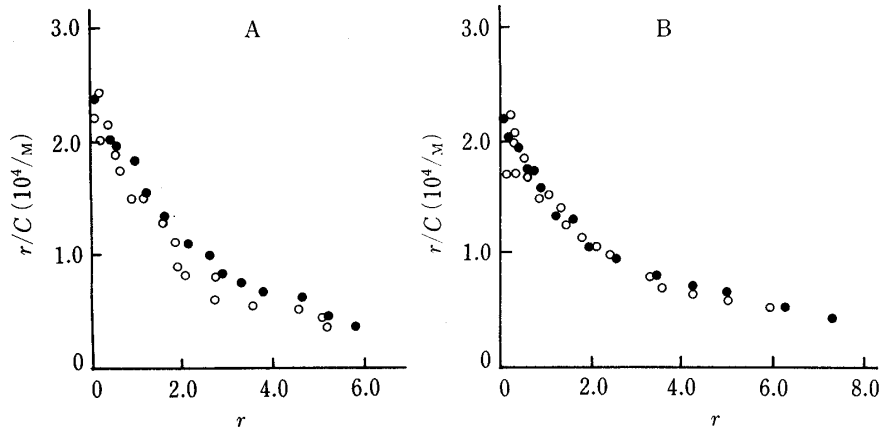

Fig. 5. Scatchard Plots for the Binding of HABCA to Serum Albumin

HABCA bindings to BSA (A) and HSA (B) were measured at 15 °C by the UC method (●) and ED method (○). Concentrations of BSA and HSA were 5.0×10^{-5} M.

Effect of Albumin Concentration on the Scatchard Plot for HABCA–BSA Binding Determined by the UC Method

HABCA binding was measured by the UC method. Figure 4 shows the Scatchard plots. At three levels of BSA, no differences were seen in the plots of HABCA binding. Thus, in the concentration range under consideration, no dependency of the plots on BSA concentration was apparent in HABCA binding. It was also observed that the UC method showed a high degree of reproducibility in measurements over a wide range of HABCA concentrations.

Comparison of the UC Method and ED Method

These two methods were compared for application to the study of drug–BSA and drug–HSA bindings. The binding data obtained by the two methods were analyzed by Scatchard plotting (Figs. 5—8) and the binding parameters were calculated by means of Karush's formula and the stepwise model. The results are shown in Tables I and II.

ED experiments were repeated five times, and the Scatchard plots show the mean values. Nonetheless, some scatter was seen in the plots for HABCA (Fig. 5) and salicylate (Fig. 7) measured at low concentrations by the ED method, whereas good agreement of the plots was observed between the two methods at high concentrations. For 2-naphthoate (Fig. 6) and warfarin (Fig. 8), data obtained by the ED method were also scattered at low concentrations.

Parameters calculated by means of Karush's formula (Table I) showed some differences in n_2 for salicylate–BSA and warfarin–HSA and in k_1 for 2-naphthoate between the two

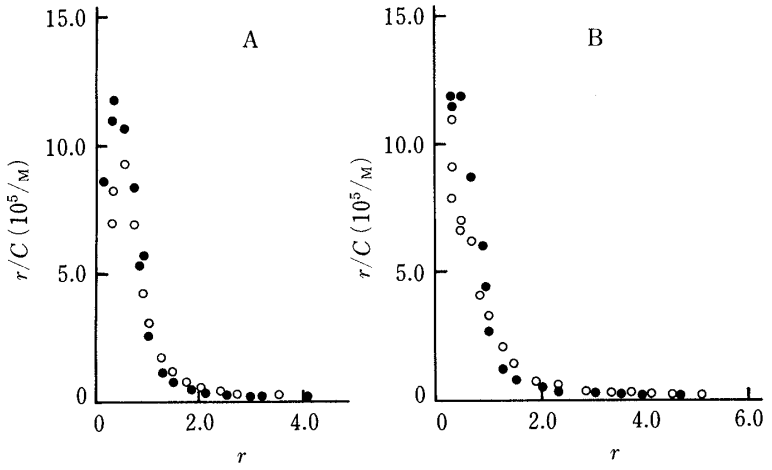

Fig. 6. Scatchard Plots for the Binding of 2-Naphthoate to Serum Albumin

2-Naphthoate bindings to BSA (A) and HSA (B) were measured at 15 °C by the UC method (●) and ED method (○). Concentrations of BSA and HSA were 5.0×10^{-5} M.

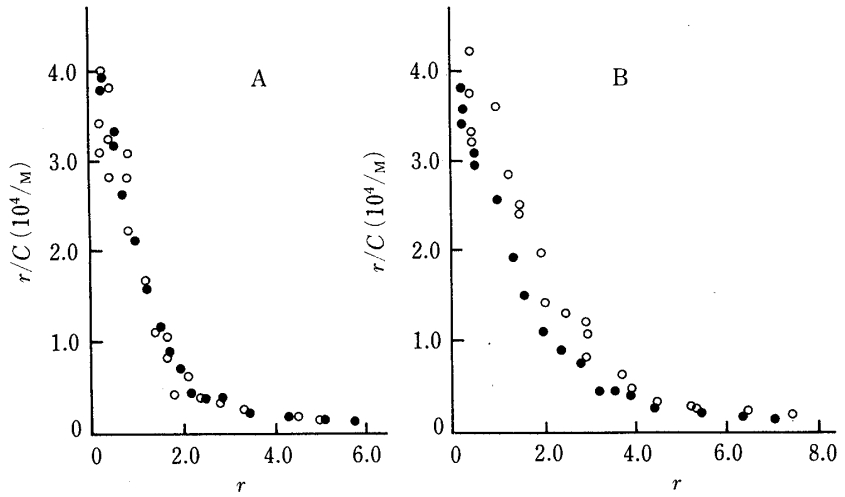

Fig. 7. Scatchard Plots for the Binding of Salicylate to Serum Albumin

Salicylate bindings to BSA (A) and HSA (B) were measured at 15 °C by the UC method (●) and ED method (○). Concentrations of BSA and HSA were 5.0×10^{-5} M.

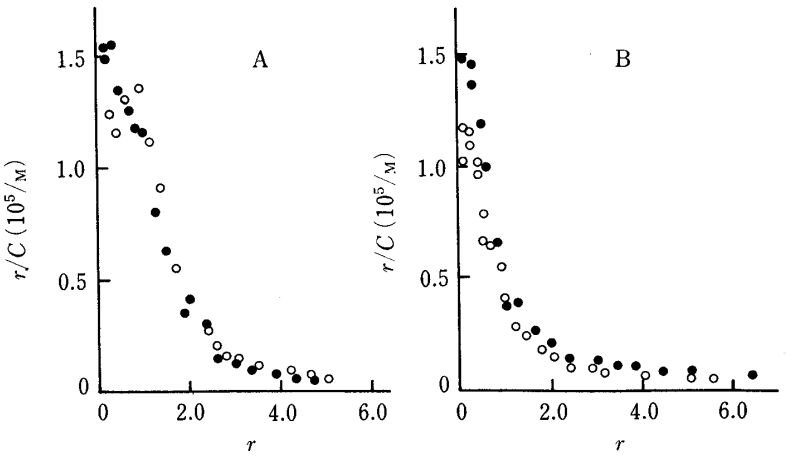

Fig. 8. Scatchard Plots for the Binding of Warfarin to Serum Albumin

Warfarin bindings to BSA (A) and HSA (B) were measured at 15 °C by the UC method (●) and ED method (○). Concentrations of BSA and HSA were 5.0×10^{-5} M.

TABLE I. Binding Parameters Calculated by Using the Karush Equation

Drug	Method	BSA				HSA			
		n_1	k_1 (10^5 M^{-1})	n_2	k_2 (10^3 M^{-1})	n_1	k_1 (10^5 M^{-1})	n_2	k_2 (10^3 M^{-1})
HABCA	UC	1.12	0.16	7.49	1.05	0.88	0.18	12.75	0.65
		(0.60)	(0.02)	(0.19)	(0.00)	(0.04)	(0.00)	(0.20)	(0.00)
	ED	1.21	0.18	10.02	0.52	1.86	0.09	13.43	0.38
		(0.59)	(0.02)	(12.97)	(0.00)	(0.32)	(0.01)	(13.24)	(0.00)
Warfarin	UC	2.03	0.91	4.87	1.15	1.18	1.45	10.50	0.85
		(0.03)	(0.02)	(10.28)	(0.00)	(0.02)	(0.19)	(1.05)	(0.00)
	ED	2.05	0.93	4.94	1.40	1.40	0.83	19.73	0.25
		(0.05)	(0.04)	(0.33)	(0.00)	(0.04)	(0.07)	(13.93)	(0.00)
2-Naphthoate	UC	1.31	12.91	4.80	2.31	1.24	15.36	5.04	4.41
		(0.00)	(3.04)	(0.35)	(0.00)	(0.01)	(7.63)	(0.05)	(0.00)
	ED	1.45	7.68	4.72	2.61	1.42	7.67	5.71	4.76
		(0.01)	(1.37)	(0.32)	(0.00)	(0.01)	(1.18)	(0.05)	(0.00)
Salicylate	UC	1.86	0.22	14.81	0.08	2.71	0.12	17.85	0.07
		(0.03)	(0.26)	(42.19)	(0.00)	(0.04)	(0.03)	(41.83)	(0.00)
	ED	1.80	0.24	31.98	0.03	2.65	0.19	15.18	0.13
		(0.05)	(0.57)	(416.4)	(0.00)	(0.10)	(0.24)	(48.65)	(0.00)

Figures in parentheses indicate the 95% confidence interval.

TABLE II. Stepwise Equilibrium Constants (M^{-1})

Drug	SA	Method	K_1 (10^5)	K_2 (10^4)	K_3 (10^3)	K_4 (10^3)	K_5 (10^3)	K_6 (10^3)	K_7 (10^3)
HABCA	BSA	UC	0.28	0.67	3.23	1.91	1.21		
	BSA	ED	0.29	0.51	2.46	1.49	0.99		
	HSA	UC	0.23	0.63	3.30	2.09	1.45	1.05	0.78
	HSA	ED	0.21	0.73	3.28	1.91	1.26	0.90	0.67
Warfarin	BSA	UC	1.93	5.11	5.28	2.21	1.12		
	BSA	ED	2.02	5.39	6.57	2.78	1.41		
	HSA	UC	2.24	0.97	4.45	2.65	1.74	1.19	
	HSA	ED	1.76	0.57	2.75	1.73	1.23	0.92	
2-Naphthoate	BSA	UC	21.39	1.86	7.01	4.09			
	BSA	ED	17.83	2.44	9.20	3.12			
	HSA	UC	26.59	2.82	1.13	5.66	2.83		
	HSA	ED	16.91	4.67	1.89	9.47	4.74		
Salicylate	BSA	UC	0.37	0.98	1.04	0.51	0.32	0.22	
	BSA	ED	0.37	0.97	0.94	0.47	0.31	0.23	
	HSA	UC	0.31	0.11	4.02	1.00	0.52	0.34	0.24
	HSA	ED	0.44	0.15	5.75	1.44	0.75	0.48	0.38

methods. Apart from these values, little or no difference was seen in any of the parameters, but the 95% confidence limit was better for the UC method. As regards K_i calculated by using the stepwise model (Table II), a difference between the two methods was seen only for 2-naphthoate, and little or no difference was recognized in parameters obtained for other drugs. Thus, the UC method and ED method gave consistent results in terms of the parameters calculated by means of Karush's formula and the stepwise model, which are currently used extensively.

The stepwise model is sufficiently general to account for cooperativity and other phenomena that may be associated with binding. The constant for binding of the first

molecule of 2-naphthoate is a hundred times or more greater than that for binding of a second molecule. In contrast, the second and third association constants are not so different, suggesting that these two binding sites may be similar. On the other hand, an advantage of Scatchard analysis is that the total number of albumin binding sites can be estimated by extrapolation even when the range of experimental data is limited. Moreover, comparison of binding ability among drugs can be done simply by the use of the Scatchard analysis.

The comparison of the two methods, UC and ED, for the determination of binding with BSA and HSA revealed some advantages and drawbacks for each method. Namely, the UC method has a high degree of reproducibility and is technically simple. However, it is not applicable to measurements of drugs with high molecular weight. On the other hand, the ED method using a dialysis cell is advantageous in that it can treat a number of specimens at one time. However, it lacks reproducibility for measurements at low drug concentrations. In conclusion, we consider that the UC method is a useful tool for studies of protein bindings; the UC method is comparable to the ED method in terms of reliability, and superior in terms of reproducibility.

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