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STUDY OF BINDING OF WARFARIN TO SERUM ALBUMINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The binding of warfarin to human serum albumin and bovine serum albumin, respectively, was studied by high-performance liquid chromatography (HPLC). Based upon the Hummel–Dreyer method, two techniques were developed: the internal calibration and the external calibration. The results obtained by the HPLC method and those obtained by the classical method (equilibrium dialysis) were compared. The external calibration method seems to be superior to others for its simplicity, speed and convenience.

INTRODUCTION

The most commonly used method for the study of the binding parameters (*e.g.*, $\bar{r}$, the average number of moles of ligand bound per mole of macromolecule, n_i, the number of binding sites, and K_i, the binding constant) is equilibrium dialysis. The method, while reliable, suffers from two serious disadvantages: (1) it takes a long time to reach equilibrium condition which possibly results, *e.g.* in denaturation of protein; (2) the equilibrium reached is in a static condition (hence, static equilibrium) which is different from the natural conditions found in a living organism (*e.g.* human body).

A simple method, analogous in principle to equilibrium dialysis, which was proposed more than twenty years ago and is now called the Hummel–Dreyer method[1], seems to overcome both disadvantages. It is rapid, *i.e.*, each binding experiment as reported in this paper is usually finished in 10 to 30 min, and the eluent is a ligand solution, thereby maintaining a dynamic equilibrium, similar to what actually occurs in living systems.

Fairclough and Fruton[2] were among the earlier users of the Hummel–Dreyer method for the study of interaction between peptide and protein. They used the conventional liquid chromatography (gel filtration) to collect the fractionated sam-

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ples for analysis. More recently Sebille *et al.*[3,4] adopted the method in high-performance liquid chromatography (HPLC). They developed two techniques in determining the values of $\bar{r}$: one derived from Hummel–Dreyer's original proposal, the internal calibration, and the other, equilibrium saturation concentration.

The work described in this paper is an attempt to extend and improve, if possible, both the work of Fairclough and Fruton[2] and the work of Sebille *et al.*[3,4] Warfarin–human serum albumin (HSA) system was chosen for study because of its richness in information in the literature[5–9]. The binding of warfarin to bovine serum albumin (BSA) was also studied for comparison. Since bovine serum albumin has been found to have a great similarity to human serum albumin in terms of molecular weight, size, shape and behavior, and since it is a standard protein in research in almost every area in biological science, we felt that the HPLC method should be tested with this protein.

Measurements are based on two techniques. One is the internal calibration method. Our approach is, however, different to a certain extent from our predecessors. The second technique is what we term "the external calibration method" which was developed in this laboratory. We describe every step of each method in detail so that the work can easily be followed and checked. As will be seen, the external calibration method is much simpler than the internal calibration method both in speed and in experimental procedure. It also saves on materials, particularly the costly proteins.

EXPERIMENTAL

Materials

Warfarin was obtained as a gift from Mr. Joseph Wong of Dupont Laboratories. Human serum albumin (lot No. 102F-9395) and bovine serum albumin (lot No. 22F-9340) were obtained from Sigma . Sodium hydrogen phosphate and sodium dihydrogen phosphate were reagent grade of J. T. Baker. All the chemicals were used as received. Glass distilled water was used in all the experiments.

Preparation of solution

The buffer was prepared by mixing an appropriate portion of aqueous sodium hydrogen phosphate and sodium dihydrogen phosphate solution controlled by a pH meter to obtain pH value 7.4. For example, 28.4 g of Na_2HPO_4 and 27.6 g of $NaH_2PO_4 \cdot H_2O$ each was dissolved in 1000 ml of glass distilled water separately. To 1000 ml of Na_2HPO_4 solution was added $NaH_2PO_4 \cdot H_2O$ solution until pH was adjusted to 7.4. This was 0.2 *M* phosphate buffer stock solution. It was then diluted to 0.067 *M* by adding water for use in our experiments.

The stock solution for the mobile phase was prepared by dissolving 57.0 mg of warfarin in 2.00 l of buffer (0.067 *M* phosphate, pH 7.4). It was then diluted to several different concentrations by adding appropriate amounts of buffer to a fixed amount of the stock solution.

The protein stock solution was prepared by dissolving 39.3 mg of protein in 10.0 ml of buffer. The exact albumin concentration was determined spectrophotometrically using the value of $E_{1\,cm}^{1\%} = 6.67$ at 279 nm. Seven different samples were then prepared according to Table I. Samples A through E were used for internal

TABLE I

PREPARATION OF PROTEIN SAMPLES (UNIT μl)

Sample	*Protein stock solution (114.0 μM)*	*Warfarin stock solution (86.3 μM)*	*Phosphate buffer (0.067 M, pH 7.4)*
A	500	125	625
B	500	250	500
C	500	375	375
D	500	500	250
E	500	750	0
F	0	0	1250
G	500	0	750

calibration; F and G for external calibration. When needed for more experimental points, they were further diluted by adding buffer solutions or their concentrations were increased by adding more concentrated protein solutions.

In all calculations molecular weights for warfarin was taken as 330.3 and serum albumin (BSA as well as HSA) as $6.9 \cdot 10^4$.

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 and a recorder. (All the experiments were carried out before we had a Hewlett-Packard integrator available. Here the area of a trough was read directly on the chromatogram using a K and E planimeter.)

The column used was Waters I-125 (30 cm × 7.8 mm I.D., particle size 37–53 μm). In most runs the back pressure was no more than 500 p.s.i. at the flow-rate 2 ml/min. However, when the concentration of warfarin in the mobile phase is high (*i.e.* using the stock solution of warfarin), the back pressure could run as high as 1000–1500 p.s.i. at the same flow-rate 2.0 ml/min.

RESULTS

Fig. 1 shows a typical chromatogram of the binding of warfarin to human serum albumin. The fact that there exists a peak and a trough demonstrates the existence of an equilibrium between $n\mathrm{L} + \mathrm{M}$ and L_n–M as expressed in the equation

$$n\mathrm{L} + \mathrm{M} \rightleftarrows \mathrm{L}_n\text{–}\mathrm{M} \quad (1)$$

where n is the number of moles, L is the ligand, M is the macromolecule and L_n–M is the ligand–macromolecule complex. The base line represents the equilibrium as "observed" by the detector, *i.e.*, UV at 313 nm where the absorption is attributed to warfarin alone. The area of the peak is a measure of the concentration of L_n–M complex, whereas the area of the trough is a measure of the concentration of warfarin equivalent to the amount that binds the protein, *i.e.*, area of trough $\cong [\mathrm{L}]_b$. Our principal task, therefore, is to find a convenient way to evaluate $[\mathrm{L}]_b$ from the area of the trough. It should be pointed out that to relate the area to concentration is a

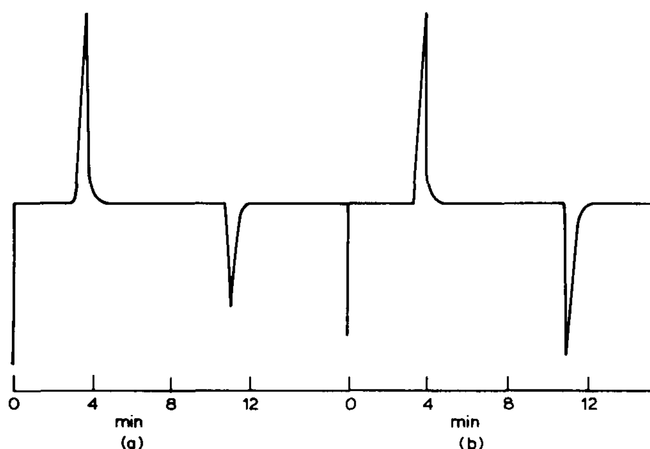

Fig. 1. Chromatograms of binding of warfarin to HSA. Detector: UV at 313 nm, 0.4 a.u.f.s. Flow-rate: 2.0 ml/min. Chart speed: 5.0 cm/10 min. Mobile phase: 37.0 μM warfarin in 0.067 M sodium phosphate buffer, pH 7.4. Sample: 50 μl (a) sample E, (b) sample D (see Table II).

standard method employed in the determination of molecular weight and diffusion coefficient with analytical ultracentrifuge. The area of the trough itself can be measured, as mentioned before, by a planimeter; or if an integrator is available, it will be automatically printed.

Internal calibration method

For each of the mobile phases with known concentrations of warfarin, *e.g.* 86.3 μM, we injected 5 samples (A–E in Table I) to the column, respectively. Five chromatograms similar to that of Fig. 1 were obtained. The area of the trough of each chromatogram was measured. The amount of warfarin in each injected sample and the amount of the protein which was kept constant was calculated. For example, in sample A (see Table II) the concentration of warfarin was 8.63 μM and that of protein 22.8 μM. Thus, in 50 μl of the sample which was injected to the column, the

TABLE II

AN EXAMPLE OF INTERNAL CALIBRATION

Mobile phase, 86.3 μM warfarin in 0.067 M phosphate buffer, pH 7.4.

Sample	*Area of trough (× 100 in.^{2})*	*Warfarin in 50 µl sample*		*HSA in 50 µl sample*	
		Conc. (µM)	*Absolute amount (µmole × 10^3)*	*Conc. (µM)*	*Absolute amount (µmole × 10^3)*
A	24.0	8.63	0.43	22.8	1.14
B	21.5	17.26	0.86	22.8	1.14
C	20.0	25.92	1.30	22.8	1.14
D	18.5	34.51	1.73	22.8	1.14
E	15.5	51.77	2.59	22.8	1.14

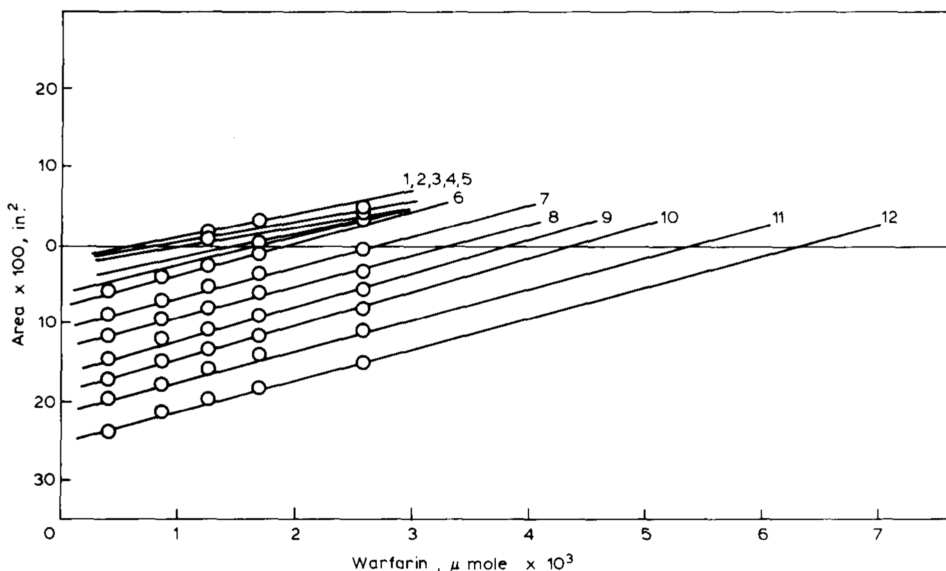

Fig. 2. Internal calibration for the determination of warfarin in μmole that binds to human serum albumin. The number next to the line indicates the concentration of warfarin in μM in the mobile phase: (1) 0.12; (2) 0.36; (3) 0.60; (4) 0.85; (5) 1.2; (6) 12.1; (7) 24.6; (8) 38.4; (9) 49.3; (10) 60.6; (11) 73.9; (12) 86.3.

absolute amounts of the substances were: warfarin $0.432 \cdot 10^{-3}$ μmole, protein $1.14 \cdot 10^{-3}$ μmole.

Table II gives as an example of a set of data for internal calibration. Fig. 2 shows the internal calibration lines for the warfarin binding to HSA. The interpolated point, *i.e.* the interception at area (= 0) of each line is taken as L_b, the amount of warfarin in μmole that binds the protein. The absolute amount of protein, *m*, in each

TABLE III

BINDING OF WARFARIN TO HSA (INTERNAL CALIBRATION METHOD)

$[L]_f$* (μM)	L_b (μmole × 10^3)	$\bar{r} = \frac{L_b}{m^{**}}$	$\frac{\bar{r}}{[L]_f} \times 10^6$ (M^{-1})
86.3	6.35	5.57	0.07
73.9	5.35	4.69	0.06
60.6	4.37	3.83	0.06
49.3	3.86	3.39	0.07
38.4	3.35	2.94	0.08
24.6	2.73	2.39	0.10
12.1	1.8	1.58	0.13
1.2	0.7	0.61	0.50
0.85	0.65	0.57	0.67
0.61	0.55	0.48	0.80
0.36	0.45	0.39	1.10
0.12	0.36	0.31	2.50

* $[L]_f = [L]_{mobile}$.
** $m = 1.14 \cdot 10^{-3}$ μmole HSA.

TABLE IV

BINDING OF WARFARIN TO BSA (INTERNAL CALIBRATION METHOD)

$[L]_f$* (μM)	L_b (μmole × 10^3)	$\bar{r} = \frac{L_b}{m^{**}}$	$\frac{\bar{r}}{[L]_f} \times 10^6$ (M^{-1})
86.3	11.1	4.87	0.06
73.9	10.8	4.74	0.06
61.6	10.3	4.52	0.07
49.3	8.9	3.90	0.08
37.0	8.4	3.68	0.10
30.8	7.6	3.33	0.10
24.6	6.0	2.63	0.10
12.3	3.5	1.54	0.13
6.2	2.3	1.01	0.16
1.2	1.6	0.70	0.58
0.61	1.5	0.64	1.07
0.36	1.1	0.48	1.32
0.12	0.9	0.39	3.30

* $[L]_f = [L]_{mobile}$.
** $m = 2.28 \cdot 10^{-3}$ μmole BSA.

sample is known, *i.e.*, $1.14 \cdot 10^{-3}$ μmole. Thus we can calculate $\bar{r}$ by use of the equation

$$\bar{r} = \frac{L_b}{m} \tag{2}$$

Since a dynamic equilibrium exists between the warfarin in the mobile phase and the ligand–protein complex in the flow, the concentration of warfarin in the mobile phase is equivalent to the concentration of free warfarin, $[L]_f$. Thus we can also calculate the well-known ratio $\frac{\bar{r}}{[L]_f}$ in the binding equilibrium. In Table III are listed the values

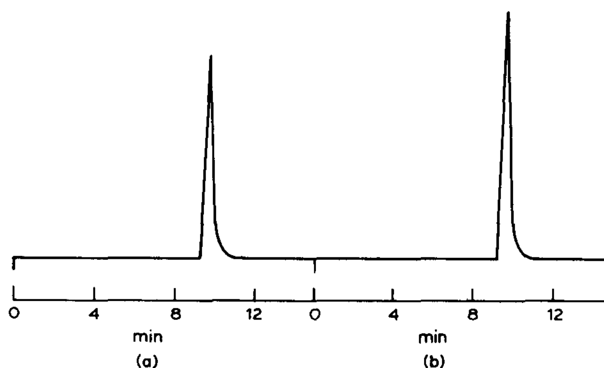

Fig. 3. Elution profile for the determination of the concentration of warfarin. Detector: UV at 313 nm, 0.4 a.u.f.s. Flow-rate: 2.0 ml/min. Chart speed: 5.0 cm/10 min. Mobile phase: 0.067 M phosphate buffer, pH 7.4. Inject: 100 μl; (a) 49.3 μM warfarin; (b) 61.6 μM warfarin.

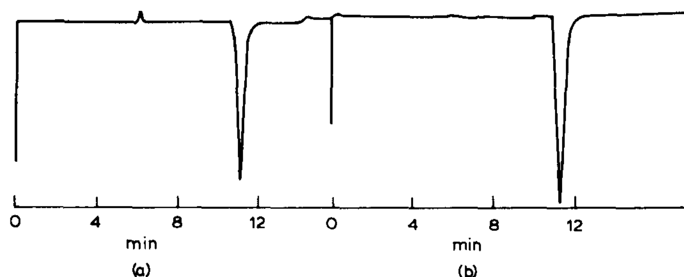

Fig. 4. Elution profile for the determination of the concentration of warfarin. Detector: UV at 313 nm, 0.4 a.u.f.s. Flow-rate: 2.0 ml/min. Chart speed: 5.0 cm/10 min. Mobile phase: (a) 37.0 μM; (b) 49.3 μM, warfarin in 0.067 M phosphate buffer, pH 7.4. Inject: 100 μl phosphate buffer.

of L_b, $\bar{r}$, and $\frac{\bar{r}}{[L]_f}$ for the binding of warfarin to HSA; in Table IV are those for the binding of warfarin to BSA.

External calibration method

A series of chromatographic experiments were run with buffer as the mobile phase and warfarin solutions (which were to be used as mobile phases for protein binding) as the injected samples. Fig. 3 illustrates the chromatogram as a function of the concentration of warfarin. Chromatographic experiments were also run for warfarin solutions as the mobile phase and buffer as the injected sample (sample F in Table I). Fig. 4 illustrates the chromatogram. It is noted that in Fig. 3 we obtain positive peaks and in Fig. 4 negative peaks (trough). Whether positive or negative, the area of the peak represents the concentration of warfarin. Within the experimental error the two sets of data are in reasonable agreement. Fig. 5 plots both sets of data.

Using each of the warfarin solutions as a mobile phase and injecting protein solution as the sample (sample G in Table I), we obtain chromatograms similar to that shown in Fig. 1. The area of trough can be directly converted into the concentration of warfarin in μM by reading the calibration plot (Fig. 5). This concentration of warfarin is $[L]_b$, the amount of ligand that binds the protein. Since the concentration of protein in the sample (*e.g.* G in Table I) injected into the column is known, [M] (the concentration of macromolecule in μM), we can calculate the value $\bar{r}$, using the equation

$$\bar{r} = \frac{[L]_b}{[M]} \tag{3}$$

As in the case of internal calibration, we use the concentration of warfarin in the mobile phase as the concentration of free warfarin in the dynamic equilibrium, $[L]_f$. Thus we can calculate the value of the ratio $\frac{\bar{r}}{[L]_f}$.

Table V gives the results of $\bar{r}$ and $\frac{\bar{r}}{[L]_f}$ for the binding of warfarin to human serum albumin and the detailed data that lead to the results of the calculations. Table VI gives the results and data for the binding of warfarin to bovine serum albumin.

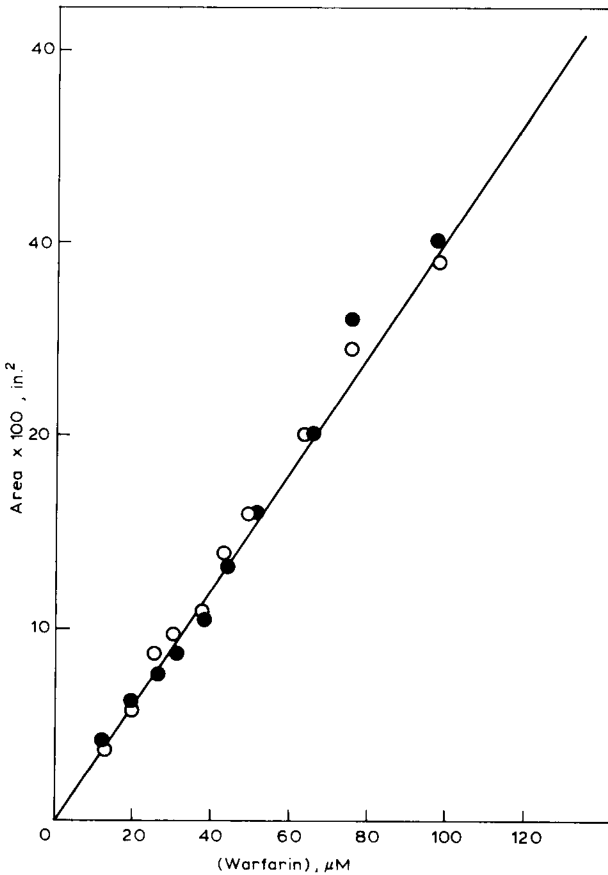

Fig. 5. External calibration plot, (○) peak; (●) trough.

TABLE V

BINDING OF WARFARIN TO HSA (EXTERNAL CALIBRATION METHOD)

$[L]_f$* (μM)	Area (× 100 in.²)	$[L]_b$ (μM)	[HSA] (μM)	$\bar{r}$	$\frac{\bar{r}}{[L]_f} \times 10^6$ (M^{-1})
86.3	22	143.4	22.8	6.28	0.07
73.9	20	130.3	22.8	5.71	0.08
60.6	17	110.7	22.8	4.85	0.08
49.3	16	97.6	22.8	4.28	0.08
38.4	13	84.5	22.8	3.71	0.10
24.6	10	64.9	22.8	2.85	0.12
12.1	7	45.3	22.8	1.99	0.16
1.2	4	12	19.2	0.63	0.52
0.6	7	22	48.0	0.46	0.76
0.3	12	38	96.0	0.39	1.30

* $[L]_f = [L]_{mobile}$.

TABLE VI

BINDING OF WARFARIN TO BSA (EXTERNAL CALIBRATION METHOD)

$[L]_f$* (μM)	Area (× 100 $in.^2$)	$[L]_b$ (μM)	[BSA] (μM)	$\bar{r}$	$\frac{\bar{r}}{[L]_f} \times 10^6$ (M^{-1})
86.3	46	119.9	22.8	5.26	0.06
73.9	42	115.8	22.8	5.09	0.07
61.6	34	99.0	22.8	4.34	0.07
49.3	32	93.4	22.8	4.09	0.08
38.4	25	75.2	22.8	3.28	0.09
30.8	21	62.6	22.8	2.75	0.09
24.6	17	57.4	22.8	2.25	0.09
12.3	13	40.2	22.8	1.76	0.14
1.2	13	41	64.0	0.64	0.53
0.6	12	38	85.3	0.35	0.74
0.3	10	32	85.3	0.38	1.25

* $[L]_f = [L]_{mobile}$.

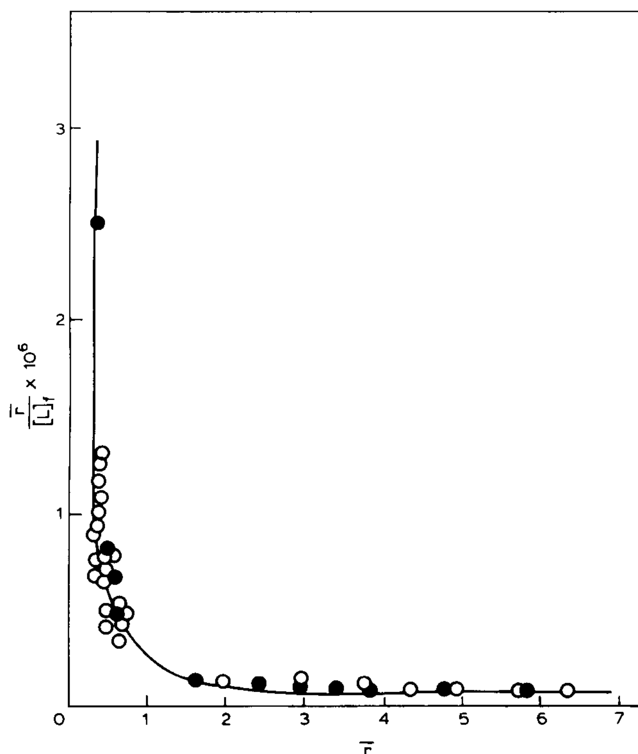

Fig. 6. Scatchard plot for the binding of warfarin by HSA. (●) Internal calibration method; (○) external calibration method.

TABLE VII

ESTIMATED BINDING PARAMETERS FOR WARFARIN BOUND TO SERUM ALBUMIN

Investigators	n_1	k_1	n_1	k_2	$\sum_i n_i K_i$	*Conditions for a two binding sites analysis at pH 7.4 and experimental technique*
Sebille *et al.*[3]	1.3	$2.18 \cdot 10^5$	3.8	$4.2 \cdot 10^3$	$3.0 \cdot 10^5$	0.067 *M* phosphate buffer, 37°C, HSA, HPLC
	1.0	$2.31 \cdot 10^5$	4	$5.9 \cdot 10^3$		0.067 *M* phosphate buffer, HSA, HPLC
Sebille *et al.*[17]	–	–	–	–	$3.2 \cdot 10^5$	0.067 *M* phosphate buffer, 37°C, HSA, HPLC, retention volume of a ligand
Nakano *et al.*[18]	–	–	–	–	$1.8 \cdot 10^5$ (HSA monomer) $1.7 \cdot 10^5$ (HSA dimer)	0.05 *M* Tris buffer containing 0.1 *M* NaCl, (pH 7.83), continuous frontal affinity chromatography
Garten *et al.*[12]	2	$8.9 \cdot 10^4$	4	$6.7 \cdot 10^3$	–	0.1 *M* Tris buffer, 27°C, HSA equilibrium dialysis
Meyer and Guttman[10]	1	$6.24 \cdot 10^6$	6	$2.6 \cdot 10^3$	–	0.04 *M* phosphate buffer, 25°C, HSA, BSA ultrafiltration, equilibrium dialysis
Mais *et al.*[13]	1.5	$2.4 \cdot 10^6$	2.4	$5.6 \cdot 10^3$	–	0.067 *M* phosphate buffer, 25°C, HSA gel filtration
Oester *et al.*[11]	1.4	$2.4 \cdot 10^5$	3.7	$3.1 \cdot 10^4$	–	0.067 *M* phosphate buffer, 25°C, HSA gel filtration
Veronich *et al.*[9]	1	$1.4 \cdot 10^6$	2	$1.8 \cdot 10^4$	–	0.067 *M* phosphate buffer, 4°C, HSA equilibrium dialysis
Mean values	1.3	$5.8 \cdot 10^5$	3.7	$7.5 \cdot 10^3$	$7.8 \cdot 10^5$	
Present work						
Internal calibration, HSA	1	$2.59 \cdot 10^6$	6	$1.31 \cdot 10^4$	–	0.067 *M* phosphate buffer, 25°C, HPLC
Internal calibration, BSA	1	$2.35 \cdot 10^6$	6	$1.55 \cdot 10^4$	–	0.067 *M* phosphate buffer, 25°C, HPLC
External calibration, HSA	1	$2.49 \cdot 10^5$	4	$3.85 \cdot 10^3$	–	0.067 *M* phosphate buffer, 25°C, HPLC
External calibration, BSA	1	$3.55 \cdot 10^5$	4	$8.30 \cdot 10^3$	–	0.067 *M* phosphate buffer, 25°C, HPLC
Mean values	1	$8.6 \cdot 10^5$	5	$9.0 \cdot 10^3$	$9.0 \cdot 10^5$	

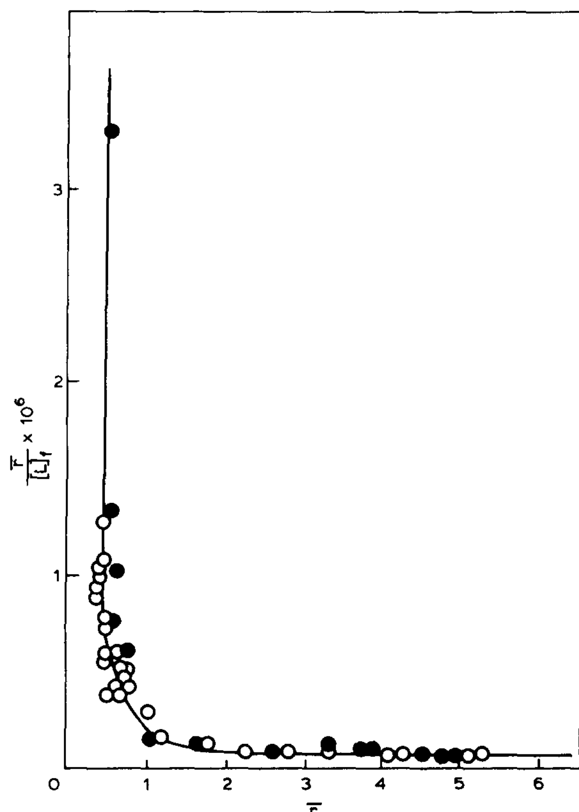

Fig. 7. Scatchard plot for the binding of warfarin by BSA. (●) Internal calibration method; (○) external calibration method.

DISCUSSION

The data in Tables III–VI (plus a few additional points to indicate uncertainties) are plotted in the form of $\frac{\bar{r}}{[L]_f}$ *vs.* $\bar{r}$ (the Scatchard plot). Fig. 6 shows the binding of warfarin to HSA; Fig. 7 shows that of warfarin to BSA. There seems to be little difference in the binding behavior of warfarin between HSA and BSA. The shape of the curve for warfarin–HSA binding is in reasonable agreement with that reported in literature, using equilibrium dialysis as the experimental method[9].

It is well established that HSA has two binding sites for warfarin

$$\bar{r} = \frac{n_1K_1[L]_f}{1 + n_1K_1[L]_f} + \frac{n_2K_2[L]_f}{1 + n_2K_2[L]_f} \quad (4)$$

We assume that BSA also has two binding sites for warfarin. Analysis of data with the curve fitting method yields the best fitting binding constants n_1, K_1 and n_2, K_2 for each protein. The results are summarized in Table VII. It is seen that there is some discrepancy in binding constants reported in literature. The discrepancy is not

entirely due to experimental error since it may also be attributed to the numerical analysis. Even the reliability and accuracy of the Scatchard plot is questionable [14]. Our calculation is based upon the computational procedures described by Thakur and Rodbard[15], also by Munsen and Rodbard[16], in which two computer steps were taken to transform input data to a B/F *vs.* F non-linear Scatchard plot.

Inspection of Table VII reveals the following findings:

(1) The mean values reported in the present work are in good agreement with the mean values reported from the analysis of the data of previous investigators.

(2) The selection of the particular serum albumin for study of warfarin binding (*i.e.* human *vs.* bovine) had little influence on the experimental results obtained.

(3) There was less variability associated with the external calibration method and the results obtained by using external calibration were in better agreement with the binding parameters reported in the literature.

Recently, two experimental methods were developed which enabled the calculation of total affinity, $\sum_i n_i K_i$, of ligand to proteins: the retention volume method[17] and the frontal affinity chromatography[18]. The former is based on the measurement of ligand retention volume under different conditions. It is an alternative to the Hummel–Dreyer method and equilibrium saturation method reported by Sebille *et al.*[3]. The latter is based on the immobilization of protein on a gel matrix. The data of total affinity for warfarin to HSA as reported in these two papers[17,18] are also included in Table VII. Both methods facilitate the checking of the reliability of n_i and K_i parameters, particularly the retention volume method which dispenses with the Scatchard plot.

In conclusion, binding study, which covers a large area in biochemistry and pharmacology, can be carried out with the HPLC method in producing reasonable results equal to the method of equilibrium dialysis. The HPLC method is fast and convenient, particularly the external calibration method.

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