Journal of Chromatography, 167 (1978) 159–170

CHROM. 11,156

STUDY OF BINDING OF LOW-MOLECULAR-WEIGHT LIGAND TO BIOLOGICAL MACROMOLECULES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

EVALUATION OF BINDING PARAMETERS FOR TWO DRUGS BOUND TO HUMAN SERUM ALBUMIN

BERNARD SEBILLE and NICOLE THUAUD

Laboratoire de Physicochimie des Biopolymères, Université de Paris XII, Avenue du Général de Gaulle, F-94010 Créteil Cédex (France)

and

JEAN-PAUL TILLEMENT

Département de Pharmacologie, Faculté de Médecine, Université Paris XII, Avenue du Général Sarrail, F-94010 Créteil Cédex (France)

SUMMARY

The binding to a biological macromolecule (human serum albumin, HSA) of small molecules (two drugs: warfarin and furosemide) has been studied by high-performance liquid chromatography. Two methods have been used and compared: frontal analysis and the Hummel and Dreyer method. The association parameters of each of the two drugs on HSA were determined. The results obtained are in good agreement with those previously published using other techniques.

The competition of these two drugs for the same site on HSA has also been shown.

INTRODUCTION

The reversible binding of a ligand to a macromolecule has been theoretically analysed by several authors[1–3], according to the "multiple equilibria theory", which yields the equation:

$$\bar{r} = \sum_{i=1}^{m} \frac{n_i K_i [A]}{1 + K_i [A]}$$

where $\bar{r}$ represents the mean number of moles of ligand bound per mole of macromolecule, m represents the number of classes of independent binding sites such that each class, i, has n_i sites with binding affinity K_i, and [A] is the concentration of unbound ligand.

This expression shows that the binding ratio $\bar{r}$ is independent of the macromolecule concentration. n_i and K_i parameters are obtained by plotting $\bar{r}/[A]$ *versus* $\bar{r}$, according to Scatchard[2]. Thus, the determination of binding parameters depends only on the measurement of $\bar{r}$ and [A].

Pharmacologists usually study the binding of drugs to proteins using equilibrium dialysis, ultrafiltration, fluorescent spectroscopy and circular dichroism methods. Filtration on Sephadex gel[4–6] is sometimes used but the low resolution of the columns limits the sensitivity of this technique. Recently (while this manuscript was in preparation) a communication about methyl orange–albumin interaction, was published by Morris and Brown[7] which dealt with the comparison of the frontal analysis chromatographic method performed at medium pressure (10 bar) with the equilibrium dialysis technique.

The present paper reports the application to high-performance liquid chromatography (HPLC) of the two methods previously described for Sephadex gel: frontal analysis[4] and the Hummel and Dreyer method[8] the principles of which are explained later.

In frontal analysis, a large sample of the mixture ligand–macromolecule is continuously applied to a size-exclusion column in order to achieve a steady state concentration on the column and is then eluted by a buffered eluent.

The chromatogram obtained shows three zones: the first corresponds to the free protein, the second to the mixture of protein-bound drug complex and excess ligand and the third corresponds to the free ligand. Thus, the height of the third plateau provides a direct evaluation of the free ligand concentration in the sample applied. The binding ratio $\bar{r}$ may be easily found by dividing the difference between total and free drug concentrations by the total protein concentration applied.

The Hummel and Dreyer method[8] involves the equilibration of a size-exclusion column with a ligand solution. A small amount of protein is injected onto the column and then eluted by the ligand solution used to equilibrate the column. The chromatogram obtained by further detection of the eluate shows a protein–ligand complex peak at the retention time of the protein followed by a negative peak at the retention time of the ligand, which represents the amount of ligand bound to the protein. The value of $\bar{r}$ can then be easily obtained.

This report deals with the binding of two drugs (the anticoagulant warfarin and the diuretic furosemide) to human serum albumin (HSA). First, both methods described above were applied and compared for the evaluation of warfarin–albumin binding parameters. Then, the Hummel and Dreyer method was applied to study furosemide binding and the competition between warfarin and furosemide for binding sites on albumin.

EXPERIMENTAL

Materials

HSA was from Sigma, St. Louis, Mo., U.S.A. (A 1887, essentially fatty acid-free albumin). Warfarin, as sodium salt, was a gift from Morell–Toraude Labs. Furosemide was a gift from Hoechst, Frankfurt/M, G.F.R. All products were dissolved in a 0.067 *M* phosphate buffer, pH 7.4.

Equipment

Apparatus. A Waters Assoc. 6000 A pump and a Waters Assoc. 440 UV detector, were used for all the experiments. For competive binding measurements a Jobin & Yvon JY3 spectrofluorimetric detector was used.

The injection of large amounts (18 ml) of material, in the case of frontal analysis, was done directly by the 6000 A pump itself. A Waters Assoc. U6K injector was included in the equipment for injections of 10–20-μl samples in the Hummel and Dreyer method.

Size exclusion columns. For frontal analysis, a Waters Assoc. μBondagel column (30 cm × 3.9 mm I.D.) with 125 Å pore diameter and 5–10 μm particle diameter was used. For the Hummel and Dreyer method, a (15 cm × 4.2 mm I.D.) column was filled by a slurry-packing technique with Glycophase G™, 100 Å pore diameter, 5–10 μm particle diameter, support (Pierce, Rotterdam, The Netherlands). Glycophase is a controlled-pore glass bead support bound with a hydrophilic non-ionic carbohydrate. The columns were thermostated at 37°.

Experimental conditions

Frontal analysis. Samples: 18 ml of 2 g/l albumin–0.5–175.0 μM warfarin mixtures; flow-rate: 1.5 ml/min; pressure: 200 bar; eluent: 0.067 M phosphate buffer, pH 7.4; UV detection: 313 nm.

Hummel and Dreyer method. Samples: 10 μl of 2.13 g/l albumin solutions; flow-rate: 0.5 ml/min; pressure: 20 bar; eluents: 0.25–1000 μM warfarin or furosemide solutions in a 0.067 M phosphate buffer, pH 7.4; UV detection: 313 and 340 nm for warfarin and 280, 340 and 365 nm for furosemide; fluorimetric warfarin detection: excitation wavelength 320 nm, emission wavelength 390 nm.

Calculations

N_i and K_i parameters were calculated on a Tektronics 4051 SN computer by a method previously described[9].

RESULTS AND DISCUSSION

Warfarin–HSA binding

Frontal analysis. Fig. 1 represents the elution pattern for warfarin–HSA frontal analysis monitored at 313 nm. The protein is hardly retained by the chromatographic support while warfarin is retained longer. We verified for all the samples applied that the distance between descending fronts is equal to the difference between warfarin and albumin retention times. This means that the retention of these components is not influenced by their concentrations. Thus γ plateau height gives an exact value of the unbound warfarin concentration [A] of the sample applied to the column. The binding ratios, $\bar{r}$ have been calculated and the classical Scatchard plot drawn as represented in Fig. 2. n_i and K_i values are given in Table I. We could not obtain low affinity sites because for this determination higher warfarin concentrations are needed and we noticed that, probably due to adsorption phenomena, efficiency of the column was greatly decreased. Even when diluted solutions of warfarin were used, the efficiency of the column was strongly reduced after about 30 experiments.

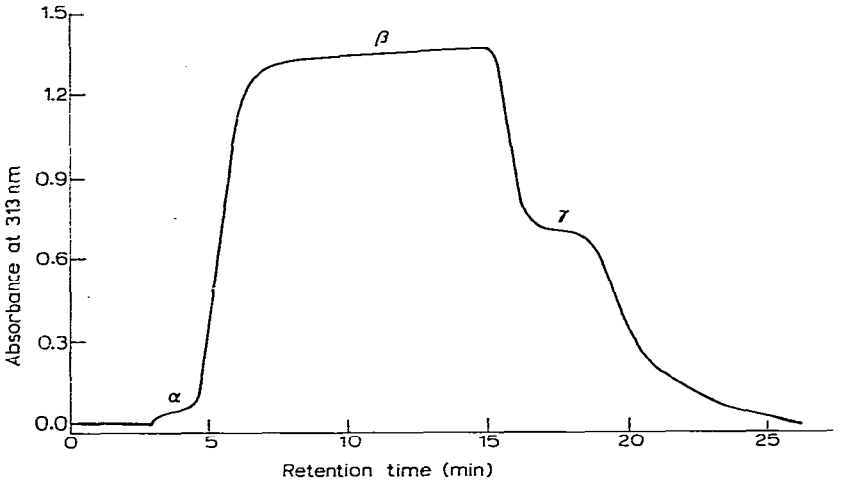

Fig. 1. Elution profile in frontal analysis of an 18-ml mixture of warfarin (10^{-4} *M*) and HSA (2 g/l). Eluent: phosphate buffer, pH 7.4 (0.067 *M*). Flow-rate: 1.5 ml/min. Temperature: 37°. Support: μBondagel, 125 Å.

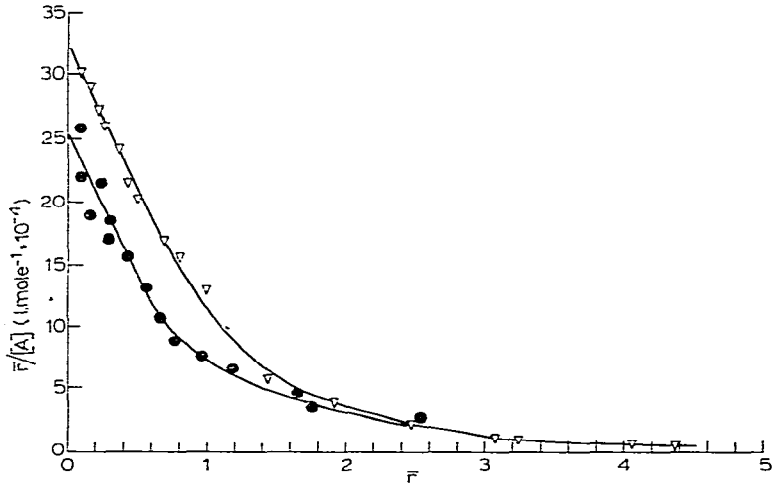

Fig. 2. Scatchard plot illustrating the binding of warfarin to HSA in phosphate buffer (pH 7.4; temperature 37°). ●, obtained by frontal analysis; ▽, obtained by Hummel and Dryer method.

TABLE I

WARFARIN–HSA BINDING PARAMETERS

n_1	K_1	n_2	K_2	*Reference*
1.16 ± 0.2	$2.1 \cdot 10^5$	—	—	This work (frontal analysis)
1.31 ± 0.2	$2.18 \cdot 10^5$	3.75 ± 0.2	4200	This work (Hummel and Dreyer method)
0.95 ± 0.2	$2.31 \cdot 10^5$	3.69 ± 0.64	5900	9
1.38	$2.03 \cdot 10^5$	3.73	2200	6

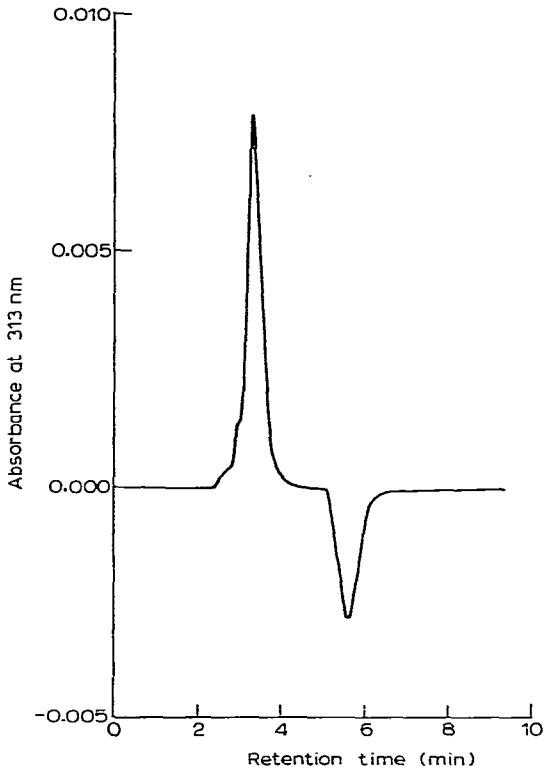

Fig. 3. Hummel and Dreyer type chromatogram obtained for sample injection of 12.5 μl of HSA solution (2 g/l) in eluting solution. Eluent: warfarin solution $0.5 \cdot 10^{-6}$ *M* in phosphate buffer (pH 7.4). Flow-rate: 0.5 ml/min. Pressure: 20 bar. Temperature: 37°. Support: Glycophase G, 100 Å.

The Hummel and Dreyer method. In order to avoid the problems mentioned above, we did not use a μBondagel column but a Glycophase one. Fig. 3 represents the shape of elution pattern obtained by this technique. The elution time of the warfarin peak depends on the warfarin concentration of the eluent (Fig. 4). This phenomenon is more pronounced at low drug concentrations. It means that the mechanism of retention of the drug on the Glycophase column is not only a size-exclusion phenomenon but that a reversible absorption may also occur. Most chromatograms were monitored at 313 nm, near warfarin maximum UV absorption wavelength. At this wavelength, the warfarin–albumin complex absorbs as much as the sum of albumin plus warfarin (isobestic point, Fig. 6). Thus the increase of protein–ligand complex peak area is identical with the negative peak area. This property provides two independent measures of the amount of bound warfarin. Eluents with warfarin concentrations higher than 10^{-4} *M* saturated the detector, so corresponding chromatograms were monitored at 340 nm. At this wavelength, only the area of the negative peak might be used for determination of the bound drug; more exact measurements are obtained when using the internal calibration method described by Hummel and Dreyer[8]. By plotting the area of the ligand peak (positive or negative) *versus* the

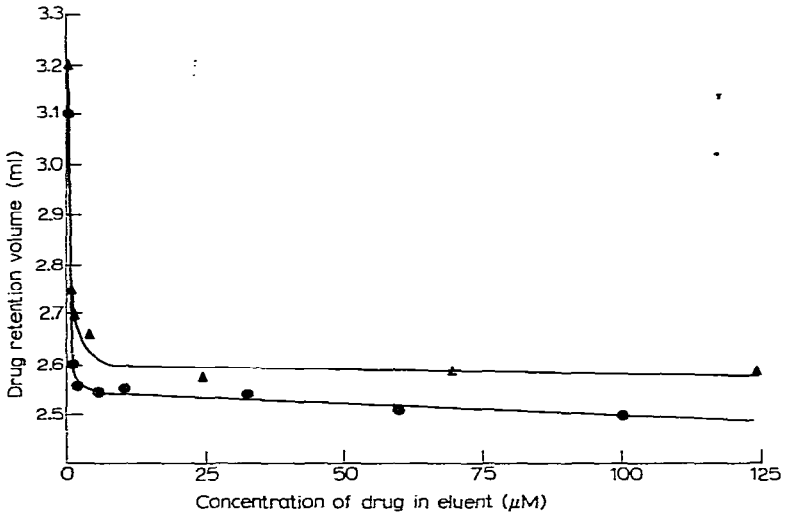

Fig. 4. Variation of drug retention volume with the eluent drug concentration. ▲, Warfarin; ●, furosemide.

number of moles of warfarin added to a constant amount of protein sample, and by interpolating it to zero, the corresponding excess of warfarin needed equals the exact amount of warfarin bound to the protein (Fig. 5). The binding ratio $\bar{r}$ thus determined facilitates drawing of the Scatchard plot represented in Fig. 2 and calculation of n_i and K_i values (Table I).

The Scatchard plots obtained by both methods (Fig. 2) have similar shape, but there are some differences in $\bar{r}/[A]$ values. An explanation of the difference found may be that it is due to an over-estimation of the free warfarin concentrations in

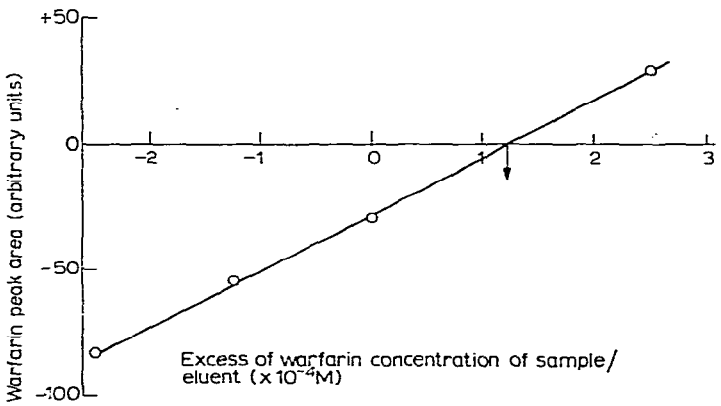

Fig. 5. Internal calibration for binding of warfarin to HSA. Peak area (at 313 nm) as a function of the excess (relative to eluent concentration) of warfarin injected with $3.2 \cdot 10^{-10}$ mole of HSA into the column. Eluent: $5 \cdot 10^{-5}$ *M* warfarin in phosphate buffer (pH 7.4).

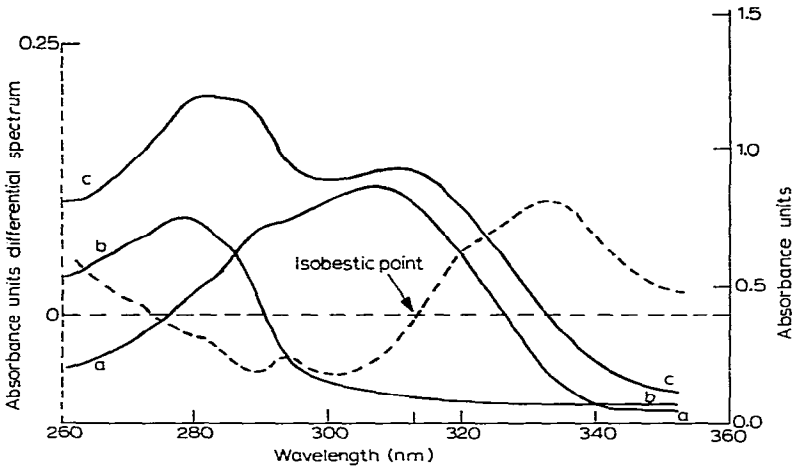

Fig. 6. UV spectra in 5-mm cells of (phosphate buffer solutions): (a) warfarin (10^{-4} *M*); (b) human serum albumin (2.13 g/l); (c) warfarin (10^{-4} *M*)–HSA (2.13 g/l) mixture. Broken line, differential spectrum between mixture (c) and solutions (a) and (b) for same path length.

the frontal analysis technique. The γ plateaux of the corresponding chromatograms are not exactly horizontal. This is probably due to the trailing front of emerging protein, and may lead to a systematic error of [A] value.

This fact may also explain the less important dispersion of the points obtained when using the Hummel and Dreyer technique. Our results are in a good agreement with those obtained by Sephadex gel filtration[6] and equilibrium dialysis and thus show the validity of both methods. Moreover, we obtained $\bar{r}$ values as low as 0.05, while previous limiting values[6] found with the Sephadex gel technique were 0.2. Thus, the sensitivity of the HPLC method provides good precision in the evaluation of high affinity binding sites.

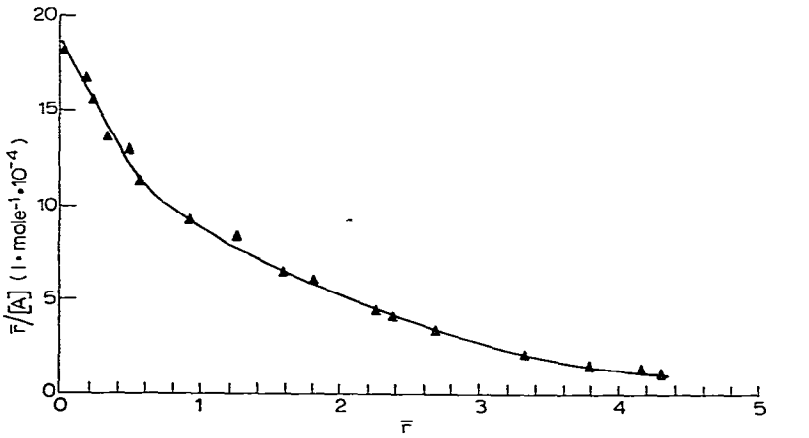

Fig. 7. Scatchard plot illustrating the binding of furosemide to HSA in phosphate buffer (pH 7.4; temperature 37°); Hummel and Dreyer method.

Furosemide binding to HSA and its competition with warfarin for binding sites

The Hummel and Dreyer method was used for these two studies.

Furosemide binding was measured in the way previously described for warfarin but the drug monitoring was performed at 280 nm for diluted solutions and 340 and 365 nm, respectively, when furosemide concentrations became higher than 100 and 350 μM. We noticed as in the case of warfarin, that the furosemide retention volume depended upon its concentration in the eluent (Fig. 4). The Scatchard plot obtained is shown in Fig. 7. Table II compares the values of n_i and K_i obtained with those obtained by equilibrium dialysis[10,11] and the ultrafiltration method[12].

TABLE II

FUROSEMIDE–HSA BINDING PARAMETERS

n_1	K_1	n_2	K_2	*Temperature (°C)*	*Reference*
0.90	$1.68 \cdot 10^5$	4.56	9600	37	This work
1.51	$6.88 \cdot 10^4$	—	—	37	10
1.42	$5.07 \cdot 10^4$	3.4	$1.58 \cdot 10^4$	37	11
0.85	$3 \cdot 10^5$	—	—	Ambient	12

Simultaneous binding of both warfarin and furosemide to HSA was measured in two sets of experiments: one with constant furosemide eluent concentration equal to 10^{-5} M and various warfarin eluent concentrations, the other one with warfarin eluent concentration equal to 10^{-5} M and various furosemide eluent concentrations. The Glycophase column could not separate the two drugs. Warfarin binding was measured by the fluorescence method, while furosemide in the range of concentrations used, gave no peak on the chromatogram. An internal calibration procedure similar to that described above and represented by a straight line in Fig. 8 was used. Simultaneous, warfarin and furosemide binding to HSA could be measured by the means of a double UV detection at 313 and 280 nm. Both drugs have different absorptions at these two wavelengths. In order to increase the sensitivity of the measurements, we used internal calibrations: we determined the amount of bound warfarin by plotting the ratio of UV peak areas measured at 280 and 313 nm *versus* the number of moles of warfarin added to the sample. The point on the curve, the ordinate of which is 4.16 (ratio between the UV absorption of furosemide measured at 280 and 313 nm) gives, from its abscissa, the amount of warfarin bound to HSA. The validity of this procedure has been verified, as shown in Fig. 8 by agreement with fluorescence measurements. We determined furosemide binding using a similar procedure (see Fig. 9), *e.g.* the point on the plotted curve the ordinate of which is 1.65 (ratio between the UV absorptions of warfarin measured at 313 and 280 nm) gives, from its abscissa, the amount of furosemide bound to HSA.

The values of warfarin and furosemide binding ratios obtained from the two sets of experiments provided the information for the part of Scatchard plots related to the high affinity binding sites as represented in Figs. 10 and 11. By comparison with plots drawn for only one drug we can conclude that each of the two drugs decreases the affinity of the primary binding sites for the other drug on albumin. Fig. 12 represents the decreased binding of one drug with increasing concentrations of the other drug in the eluent.

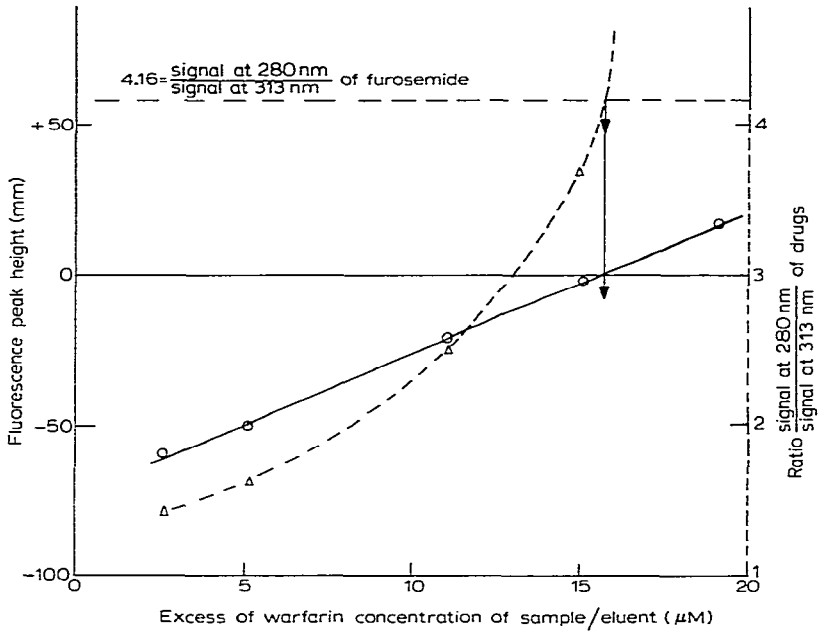

Fig. 8. Internal calibration for binding of warfarin to HSA in the presence of furosemide (10^{-5} *M*) in the eluent. △, Ratio of drug peak areas at 313 and 280 nm as a function of the excess (relative to eluent concentration) of warfarin injected with 10 μl of HSA (2.13 g/l). ○, Area of fluorescence warfarin peak as a function of the excess of warfarin injected (λ_{ex} = 320 nm, λ_{em} = 390 nm). Eluent: $5 \cdot 10^{-6}$ *M* warfarin, 10^{-5} *M* furosemide, phosphate buffer (pH 7.4).

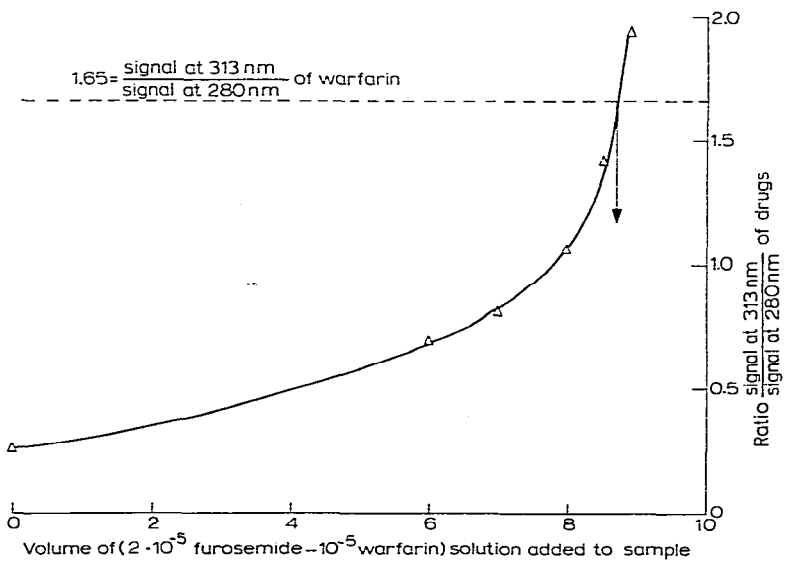

Fig. 9. Internal calibration for binding of furosemide to HSA in the presence of warfarin (10^{-5} *M*). Ratio of drug peak areas at 313 and 280 nm as a function of the excess of furosemide added to 10 μl (2.13 g/l) HSA. Eluent: 10^{-5} *M* warfarin, $5 \cdot 10^{-6}$ furosemide phosphate buffer (pH 7.4).

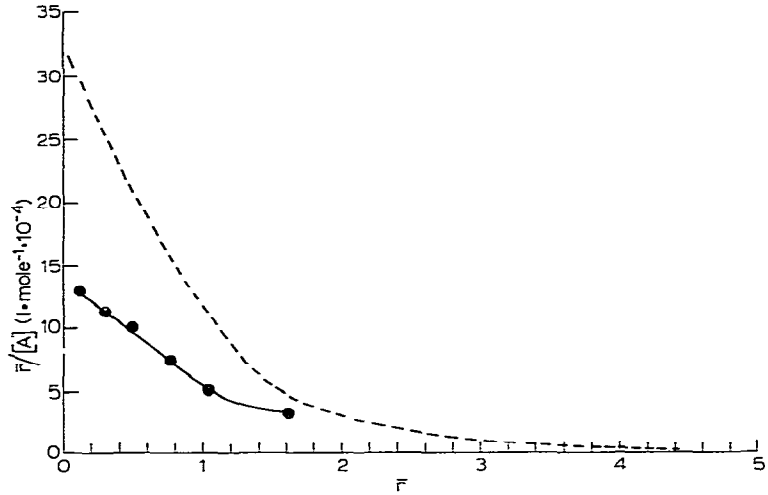

Fig. 10. Scatchard plot illustrating the binding of warfarin to HSA in the presence of furosemide (10^{-5} *M*) (●—●) and in the absence of furosemide (– – –). Phosphate buffer 0.067 *M* (pH 7.4).

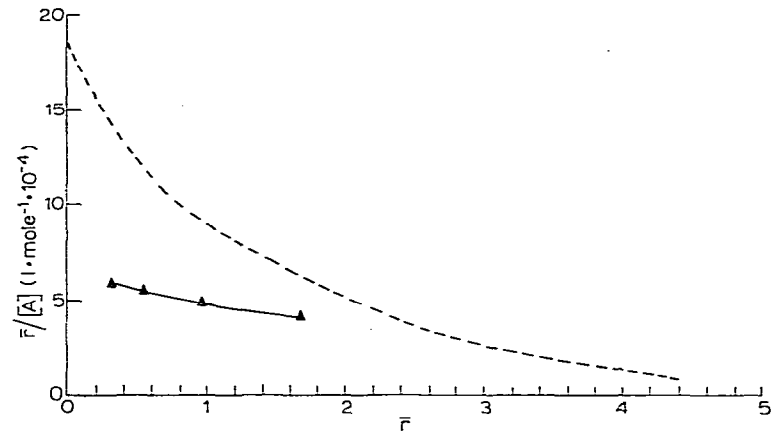

Fig. 11. Scatchard plot illustrating the binding of furosemide to HSA in the presence of warfarin (10^{-5} *M*) (▲—▲) and in the absence of warfarin (--------).

In order to establish whether the two drugs compete for the same primary binding site on the albumin molecule, we have tested the following equation proposed by Klotz *et al.*[13]

$$\frac{\bar{r}_A}{\bar{r}_B} \cdot \frac{[B]}{[A]} = \frac{K'_A}{K'_B}$$

where $\bar{r}_A$ and $\bar{r}_B$ are the binding ratios of the two competitive compounds when their free concentrations are [A] and [B], respectively; K'_A and K'_B represent their apparent primary binding site constants and are defined as the slopes drawn through

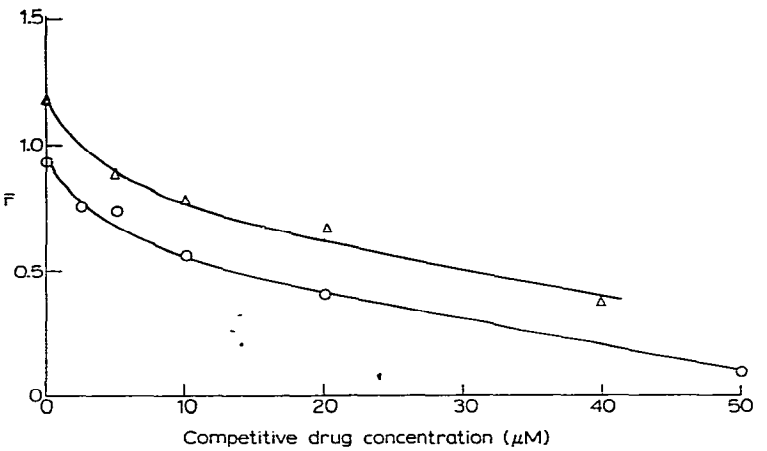

Fig. 12. Mean binding ratio, $\bar{r}$, of one drug as a function of the other drug concentration in the eluent. △, Binding of warfarin with increasing concentration of furosemide. ○, Binding of furosemide with increasing concentration of warfarin.

TABLE III

EVALUATION OF ($\bar{r}_A/\bar{r}_B$) ([B]/[A]) RATIOS IN WARFARIN–FUROSEMIDE COMPOSITION EXPERIMENTS

Mean binding, $\bar{r}_A$, for warfarin	*Mean binding, $\bar{r}_B$, for furosemide*	*Free warfarin concentration, [A] (μM)*	*Free furosemide concentration, [B] (μM)*	$\frac{\bar{r}_A}{\bar{r}_B} \cdot \frac{[B]}{[A]}$
0.285	0.753	2.5	10	1.52
0.505	0.741	5	10	1.36
1.037	0.357	20	10	1.45
0.881	0.298	10	5	1.48
0.780	0.555	10	10	1.40
0.669	0.975	10	20	1.37

the high affinity points of the respective Scatchard plots. Table III gives the values found for ($\bar{r}_A/\bar{r}_B$) ([B]/[A]) in the two sets of experiments. They are almost constants, with a mean value of 1.45 wich agrees reasonably with the 1.68 value calculated for the ratio K'_A/K'_B. This may suggest that the two drugs are both bound at the same primary binding site on HSA as demonstrated by means of fluorescent probe displacements[14] and equilibrium dialysis[10].

CONCLUSION

HPLC on a size-exclusion Glycophase G column, an adaptation of the Hummel and Dreyer method, was presented as a technique for determination of drug-albumin binding parameters. This method was compared with the frontal analysis technique on a μBondagel column and it showed that the first method allows for more complete determination of the Scatchard plot, resulting in both high and low affinity binding sites on HSA. Moreover, the use of HPLC with sensitive continuous UV detection, allowed determination of binding ratios $\bar{r}$ as low as 0.05 for warfarin

and 0.02 for furosemide, thus giving an accurate measurement of high affinity binding sites on HSA. The results were obtained in a few minutes, without the potential disadvantages of dialysis and ultrafiltration methods, such as osmotic effects, protein denaturation and binding to membranes. To prevent the latter inconvenience, the chromatographic support must be sufficiently inert for both macromolecule and ligand. The degree of binding was measured directly with no change in the equilibrium conditions. With this method, it was possible to study quickly and precisely the competitive binding of two drugs, even when they were not separated by the column.

REFERENCES

1 I. M. Klotz, *Arch. Biochem.*, 9 (1946) 109.
2 G. F. Scatchard, *Ann. N.Y. Acad. Sci.*, 51 (1949) 660.
3 R. L. Scott, *Rec. Trav. Chim.*, 75 (1956) 787.
4 G. C. Wood and P. F. Cooper, *Chromatogr. Rev.*, 12 (1970) 88.
5 L. W. Nichol, A. G. Ogston and D. J. Windsor, *Arch. Biochem. Biophys.*, 121 (1967) 517.
6 Y. T. Oester, S. Keresztes-Nagy, R. F. Mais, J. Becktel and J. F. Karolinski, *J. Pharm. Sci.*, 65 (1976) 1673.
7 M. J. Morris and J. R. Brown, *J. Pharm. Pharmacol.*, 29 (1977) 642.
8 J. P. Hummel and W. J. Dreyer, *Biochim. Biophys. Acta*, 63 (1962) 530.
9 J. P. Tillement, R. Zini, P. d'Athis and G. Vassent, *Europ. J. Clin. Pharmacol.*, 7 (1974) 307.
10 R. Zini, P. d'Athis, A. Hoarcau and J. P. Tillement, *Europ. J. Clin. Pharmacol.*, 10 (1976) 139.
11 J. Prandota and A. W. Pruitt, *Clin. Pharmacol. Ter.*, 17 (1975) 159.
12 F. Andreasen and P. Jakobsen, *Acta Pharmacol. Toxicol.*, 35 (1974) 49.
13 I. M. Klotz, H. Triwush and F. M. Walker, *J. Amer. Chem. Soc.*, 70 (1948) 2 935.
14 G. Sudlow, D. J. Birkett and D. N. Wade, *Mol. Pharmacol.*, 11 (1975) 824.