Journal of Chromatography, 204 (1981) 285–291
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,168

RETENTION DATA METHODS FOR THE DETERMINATION OF DRUG–PROTEIN BINDING PARAMETERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The binding to human serum albumin of some drugs (warfarin, furosemide and phenylbutazone) has been studied by high-performance liquid chromatography. Two methods have been used and compared, based on the measurement of the ligand retention volume under different conditions. The obtained total affinities of the drugs for the protein are in accordance with our previous results. The equilibrium saturation method leads easily to n_i and K_i parameters from the retention volume of the ligand.

INTRODUCTION

Gel filtration chromatography has been widely applied for the determination of binding parameters of macromolecules–low-molecular-weight ligands[1]. Except in the case of very stable associations, where zonal chromatography is suitable, it is necessary to use specific processes to prevent dissociation of the complex during its passage through the column.

In a previous paper[2], we have described the application of the Hummel and Dreyer method[3] with a high-performance liquid chromatograph for the determination of binding parameters of warfarin–human serum albumin. It is also possible to obtain these parameters by an equilibrium saturation method with the same apparatus[4]. In the present work, we have used another method which relies upon the determination of the retention volume of a drug eluted on an appropriate column with a protein solution. We have previously used this method in the case of L-tryptophan–human serum albumin (HSA)[5]. This method has some features similar to the work of Uekama *et al.*[6] on steroid–cyclodextrin associations and of Horváth *et al.*[7] on ion–nucleotide complexes.

We have compared the results of the equilibrium saturation method with those obtained by retention volume determination.

0021-9673/81/0000–0000/$02.50

THEORETICAL

The reversible binding of a ligand to a macromolecule is governed by the multiple equilibria theory[8–10] which yields the equation

$$\bar{r} = \sum_{i=1}^{i=m} n_i K_i [A]_f / (1 + K_i [A]_f)$$

where $\bar{r}$ is the mean number of ligands per mole of macromolecule, m is the number of classes of independent sites where each class, i, has n_i sites with binding affinity K_i and $[A]_f$ is the concentration of free (unbound) ligand. The retention volume of the ligand, V_R, is given by the usual chromatography equation

$$V_R = V_0 + K v_f$$

where V_0 is the retention volume of an unretained compound, v_f is the stationary phase volume and K is the distribution coefficient of the ligand between the stationary and mobile phases. If the mobile phase contains a polymer of concentration $[P]_0$ giving with the ligand A complexes PA, PA_2 ..... PA_n a new distribution coefficient, K', needs to be considered because of the different equilibria presented in Table I. The ligand retention volume becomes:

$$V'_R = V_0 + K' v_f$$

If we define α as the ratio of the concentration of bound ligand, $[A]_b$, to the concentration of total ligand, $[A]_0$, we obtain the following equation:

$$\alpha = [A]_b / [A]_0 = \bar{r}[P]_0 / [A]_0 \tag{1}$$

Since by definition $K' = [A]_{st}/[A]_0$ and $K = [A]_{st}/[A]_f$, where $[A]_{st}$ is the ligand concentration in the stationary phase, it follows that $K' = (1 - \alpha)K$ and:

$$\frac{V'_R - V_0}{V_R - V_0} = \frac{K'}{K} = 1 - \alpha = 1 - \frac{\bar{r}[P]_0}{[A]_0}$$

Then, since

$$[A]_0 = [A]_f + [A]_b = [A]_f \left(1 + r \frac{[P]_0}{[A]_f}\right)$$

We obtain:

$$\frac{V'_R - V_0}{V_R - V_0} = \frac{1}{1 + ([P]_0/[A]_f)} = \frac{1}{1 + \sum_{i=1}^{i=m} n_i K_i [P]_0 / (1 + K_i [A]_f)} = \frac{K'}{K} \tag{2}$$

This equation shows the dependence of the retention volume, V'_R, on the association parameters, n_i and K_i, polymer concentration, $[P]_0$, and free ligand concentration, $[A]_f$ (dependent upon the amount of injected ligand).

TABLE I

EQUILIBRIUM BETWEEN STATIONARY AND MOBILE PHASES IN THE PRESENCE OF PROTEIN

Stationary phase		*Mobile phase*
		free ligand in mobile
A_{st}	$\longrightarrow$	A_f phase
ligand in stationary	$\longleftarrow$	
phase		
		+
		P
		↑↓
		$PA + PA_2 + \ldots PA_n$
		bound ligand in mobile phase

If the amount of injected ligand is small enough to render $K_i[A]_f$ negligible compared with 1, eqn. 2 simplifies to

$$\frac{V'_{R1} - V_0}{V_R - V_0} = \frac{1}{1 + \sum_{i=1}^{i=m} n_i K_i [P]_0} = 1 - \alpha_1 = \frac{K'}{K} \tag{3}$$

where V'_{R1} and α_1 are the limit values of V'_R and α when the injected amount of ligand tends to zero.

$$\alpha_1 = \sum_{i=1}^{i=m} n_i K_i [P]_0 / 1 + \sum_{i=1}^{i=m} n_i K_i [P]_0 \tag{4}$$

Eqn. 3 leads to the "total affinity" $\sum_{i=1}^{i=m} n_i K_i$ since:

$$\sum_{i=1}^{i=m} n_i K_i = \frac{1}{[P]_0} \cdot \frac{1}{1 - \alpha_1} \tag{5}$$

For a specific value of $[P]_0$, it is possible to determine $V'_{R1} - V_0$ which is just half of $V_R - V_0$ when $\alpha_1 = 0.5$. Under these conditions

$$\sum_{i=1}^{i=m} n_i K_i = 1/[P]_0 \tag{6}$$

and we can readily obtain the total affinity.

EXPERIMENTAL

Apparatus

A 6000 A pump, U6K injector and a 440 UV detector, all from Waters Assoc. (Milford, MA, U.S.A.), were used for all the experiments. The monitored wavelength was 280 nm for furosemide and phenylbutazone and 313 nm for warfarin. The stainless-steel column (15 cm × 4.7 mm I.D.) was filled by a slurry packing technique with 10-μm LiChrosorb Diol support (E. Merck, Darmstadt, G.F.R.).

Materials

Warfarin (Merell-Toraude Laboratory, Paris, France), furosemide (Hoechst, Frankfurt/M,G.F.R.) and phenylbutazone (Ciba-Geigy, Easle, Switzerland) were gifts. HSA (A 1887, essentially fatty acid free albumin) was from Sigma (St. Louis, MO, U.S.A.). All products were dissolved in a 0.067 M phosphate buffer, pH 7.4. In all the experiments, the column temperature was maintained at 37°C.

RESULTS AND DISCUSSION

The retention volumes of some drugs injected onto a size exclusion chromatographic support eluted by different solutions of human serum albumin were measured. Fig. 1 shows the variation of retention volume with the injected amount of different drugs. As predicted by eqn. 2, the retention volume reaches a limit V'_{R1} when the injected quantity tends to zero. This limiting retention volume allows the determination of $\Sigma n_i K_i$ by using eqn. 3.

We observed that peak dissymetry grows with increasing warfarin injection (Fig. 2). This can be explained by eqn. 2 in which K' depends on the injected amount of ligand. If $K_i[A]_f$ is small enough for eqn. 3 to be valid, K' is a constant and the ligand peak is symmetrical.

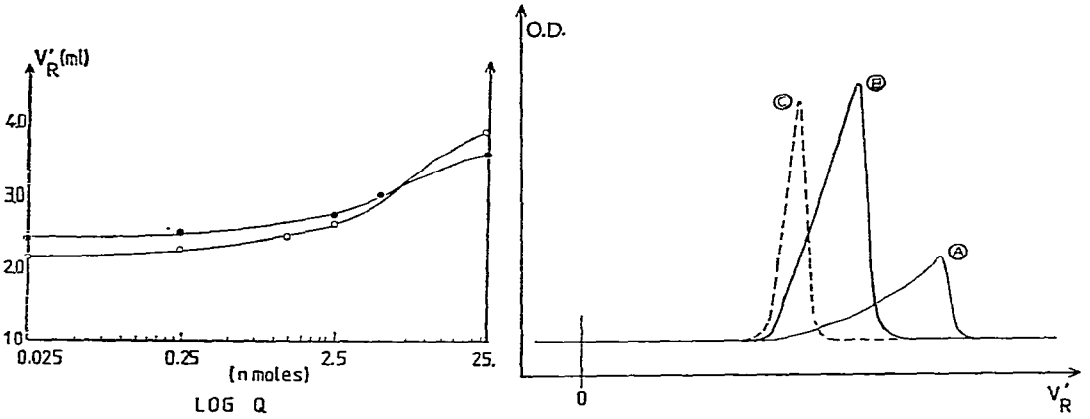
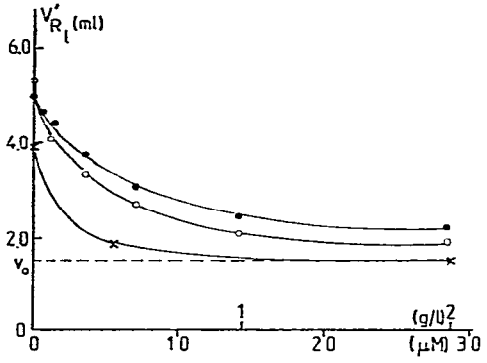


Fig. 1. Influence of injected ligand amount, Q, on retention volume. Eluent: 1 g/l HSA (buffered at pH = 7.4); flow-rate 0.4 ml/min. ○, Warfarin; ●, furosemide.

Fig. 2. Shape of chromatc grams as a function of injected ligand amount. Eluent: 2 g/l HSA (buffered at pH = 7.4); Flow-rate 0.4 ml/min. Injection (25 μl warfarin): A, 10^{-3} M, 0.5 O.D. units full scale; B, $2 \cdot 10^{-2}$ M, 0.2 O.D. units full scale; C, 10^{-5} M, 0.02 O.D. units full scale.

The variation of limiting retention volume of the ligand as a function of protein concentration is shown in Fig. 3, and it is seen that the curve has a hyperbolic profile as predicted by eqn. 3. With the V'_{R1} values and using eqn. 3 it is possible to calculate α_1 and to plot α_1 as a function of log $[P]_0$. The curve of Fig. 4 has the shape predicted by eqn. 5.

It is possible to calculate the total affinity $\Sigma n_i K_i$ either from the specific value of $1/[P]_0$ when $\alpha_1 = 0.5$, or by using eqn. 4 for each value of α_1 and different $[P]_0$.



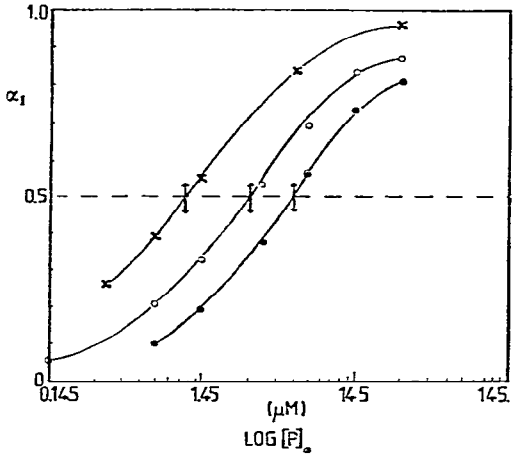


Fig. 3. Limiting retention volume, V'_{R1}, of different drugs as a function of HSA concentration in the eluent. ○, warfarin; ●, furosemide, ×, phenylbutazone.

Fig. 4. Variation of α_1 as a function of HSA concentration, $[P]_0$, in the eluent. For symbols, see Fig. 3.

In Table II, we have compared the results obtained by these different procedures for three drugs: warfarin, furosemide and phenylbutazone. The results previously reported[2,11] using Hummel and Dreyer's method are in good agreement, as were those of the equilibrium saturation method applied to warfarin–HSA binding[3].

TABLE II

TOTAL AFFINITY $\sum_{i=1}^{i=m} n_i K_i$ (M^{-1}) OF VARIOUS DRUGS FOR HSA

	Phenyl butazone	*Warfarin*	*Furosemide*
$n_1K_1 + n_2K_2$ (Hummel and Dreyer's method)	820.000 (ref. 11)	301.000 (ref. 2)	191.000 (ref. 2)
$\sum_{i=1}^{i=m} n_i K_i = 1/[P_0]$ (for $\alpha_1 = 0.5$)	860.000	320.000	180.000
$\sum_{i=1}^{i=m} n_i K_i$, mean value of $\frac{1}{[P_0]} \cdot \frac{\alpha_1}{(1-\alpha_1)}$ (from eqn. 5)	845.000	325.000	186.000

We now discuss in detail a point mentioned in our previous work on the equilibrium saturation method[3]. In this method, the eluent is a buffered solution of drug and protein. The injection of 25 μl of buffer alone leads to the appearance of two negative peaks: the first corresponds to the liganded protein and the second to the free ligand. We noticed that in this case the retention volume of the ligand, V'_R,

was dependent upon the extent of association with protein[3]. From the theoretical results mentioned above, we are now able to explain this phenomenon. Indeed, from eqn. 1, V'_R is directly related to α:

$$V'_R - V_0 = (V_R - V_0)(1 - \alpha) \tag{7}$$

In this formula, there is a linear relationship between V'_R and $(1 - \alpha)$. We have verified this by plotting V'_R as a function of $(1 - \alpha)$. The parameter α is obtained, using eqn. 1, from the measurement of ligand peak area.

The results obtained from our previous experiments[3,12] are described in Fig. 5. There is satisfactory agreement between the experimental values and those provided by eqn. 7, especially for the protein solutions at low concentrations.

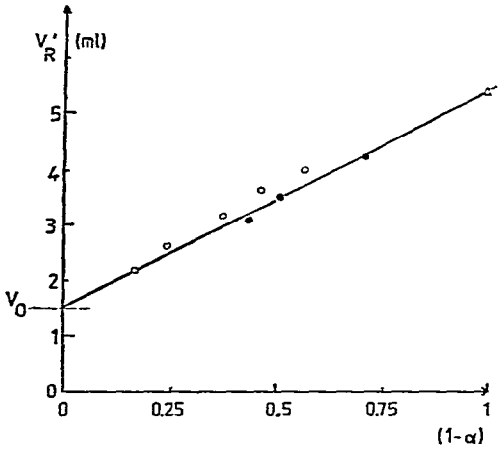


Fig. 5. Warfarin retention volume as a function of the free drug fraction, $(1 - \alpha)$, in the equilibrium saturation method. ○, 2 g/l HSA; ●, 0.4 g/l HSA; △, warfarin retention volume in the buffer alone as eluent ($\alpha = 0$).

Owing to the linear relationship between α and V'_R, it is possible to determine α from experimental V'_R values and to calculate $\bar{r}$ from eqn. 1. Then, a Scatchard plot leading to n_i and K_i parameters can be drawn[3].

It may be concluded that, in the saturation method, the mean number of bound ligands, $\bar{r}$,can be obtained either from measurement of the ligand peak area or from the ligand retention volume.

CONCLUSION

The retention volume of a ligand eluted by a protein solution depends on the association of the ligand with the macromolecules. It is possible to work out very easily the total affinity $\Sigma n_i K_i$ from the limiting retention volume, *i.e.*, the retention volume extrapolated to a zero amount of injected ligand. The results obtained for warfarin, furosemide and phenylbutazone association with HSA are in a good agreement with previous results[2,11] obtained by using Hummel and Dreyer's method.

The observed change of the ligand retention volume with extent of association has allowed us to explain some features of the equilibrium saturation method that we have previously described[3]. We have demonstrated that, in this method, the binding parameter, $\bar{r}$, and more generally the parameters n_i and K_i can be obtained from the determination of the retention volume of the ligand. This very easy procedure offers new possibilities for studying ligand–macromolecule interactions.

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