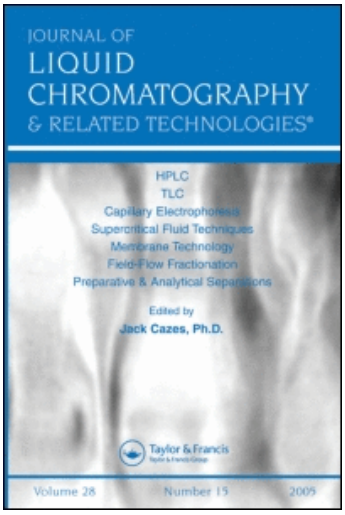

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A. Zatón[a]; A. Martinez[a]; J. M. De Gandarias[a]
[a] Departamento de Bioquimica, Colegio Eniversitario de Alava, Vitoria-Gasteiz, Spain

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STUDY OF BINDING OF BENZYL-THIOURACIL TO HUMAN SERUM ALBUMIN BY GEL FILTRATION CHROMATOGRAPHY

A. Zatón, A. Martinez, and J. M. de Gandarias
Departamento de Bioquimica
Colegio Eniversitario de Alava
Vitoria-Gasteiz, Spain

ABSTRACT

The binding of benzyl-thiouracil to human serum albumin was studied at 37°C and pH 7.4 by Sephadex filtration chromatography based upon Hummel and Dreyer's method. As the benzyl-thiouracil (ligand) was adsorbed on to the gel matrix, the free ligand concentrations had to be corrected through the ligand distribution between the stationary and mobile phases.

A good agreement was found between binding parameters - calculated by this method and by the classical method (equilibrium dialysis). Binding is characterized by one binding site with a moderate association constant ($K_1 = 5.7 \times 10^4\ M^{-1}$) and two binding sites with a low association constant ($K_2 = 7.8 \times 10^3\ M^{-1}$).

INTRODUCTION

For measurement of the binding of low-molecular-weight ligands to macromolecular compounds, gel filtration chromatography has been widely used (1,2). However, the adsorption of solute molecules on the gel in the column modifies the chromatographic parameters (3,5). If adsorption of ligand molecules takes place on the gel surface, the ligand peak height or the area un

der the peak in the chromatogram will not be proportional to the concentration of injected ligand, because of retardation of some amount of ligand molecules (5). So studies on the adsorption of the ligand samples on the gel should be made with all chromatographic methods, except that of Hummel and Dreyer (6) in which an internal or external calibration (7) is previously carried out. In this particular method (Hummel and Dreyer) the adsorption of ligand molecules on the gel beds has no influence on the determining of the binding of this ligand to a macromolecule. Therefore the binding parameters are not affected, theoretically, by adsorption of the ligand on to the gel.

Nevertheless, we determined binding parameters of different compounds with human serum albumin using several techniques, and found that in the case of some ligands, the results obtained by Hummel and Dreyer's method (6) differed from those obtained by other methods. These ligands may be the same as those which are adsorbed on to the gel. (Studies of the adsorption of several substances on to Sephadex gel seem to indicate this (3,8-12). Thus, the work described in this paper is an attempt to compare the binding parameters obtained for a ligand that is adsorbed on to the gel, by the Hummel and Dreyer method, with those obtained by the classical method (equilibrium dialysis). The benzyl-thiouracil (BTU)-human serum albumin (HSA) system was chosen for study because the BTU is adsorbed onto the Sephadex G-25 (fine) (12), gel which was also used in the Hummel and Dreyer's method (6).

EXPERIMENTAL

Human serum albumin (HSA) fraction V, essentially fatty acid-free, and 6-n-benzyl-2-thiouracil (BTU) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex gel G-25 fine grade and Blue Dextran 2000 were obtained from Pharmacia (Uppsala, Sweden). All products were dissolved in a 0.067 M phosphate buffer, pH 7.4.

Gel filtration chromatography was performed at 37°C in a Pharmacia K 16/40 glass column. The flow rate was controlled with a LKB peristaltic pump. Samples were directly monitored at 254 nm with a LKB detector. All solutions were carefully degassed before use.

The column was packed according to the instructions from the manufacturer (13). For each of the eluents with know concentrations of BTU, we injected 5 samples into the column, respectively. From the height of the second peak appearing in each chromatogram, an internal calibration was plotted, and the binding BTU concentrations were then calculated in a similar manner as has been described by Sun et al (7). The results of these experiments were processed according to Scatchard's method (14).

The adsorption experiments were carried out mixing 0.05 g of dry Sephadex with 2 ml of a known concentration of BTU. The mixture, in hermetically sealed tubes to avoid evaporation, was placed in a thermostatic bath at 37°C. After 1 h 30 min, the concentration of BTU was determined at 270 nm by a Beckman spectrophotometer. This concentration was substracted from the initial concentration of BTU (a concentration that had been obtained after the dry gel had swollen (12)), and the amount of BTU adsorbed on the gel was then calculated.

To prove if the adsorption of BTU onto the gel is a reversible process the following experiment was carried out. The adsorption of BTU on the gel was measured. Then the BTU was withdrawn completely from the gel thus eliminating the physically adsorbed molecules. After this the gel was once more submitted to adsorption by the same quantity of BTU and the adsorption was measured again.

The equilibrium dialysis was carried out at 37°C in hermetically sealed recipients. Bags were made with dialysis tubing manufactured by Union Carbide Corporation. These bags contained

5 ml of HSA (72 or 145 μM) dissolved in a known BTU concentration. The 5 ml of external solution had the same BTU concentration as the dialysis bags. Dialysis times of 3-4 h were used. Equilibrium ligand concentrations were measured in the protein free solution by spectrophotometry at 270 nm. Results were corrected for possible adsorption on cell and membrane by comparison with experiments carried out without protein. Each graph point is a mean of three experiments.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram of the binding of BTU to HSA. The first eluting peak corresponds to the ligand-macromolecule complex (BTU-HSA), and the second one to the ligand (BTU) equivalent of the amount that binds the protein.

The elution volume, Ve, of BTU-HSA complex with BTU-containing eluent is always decreased when compared with the chromatograms obtained with pure buffer as eluent. The shift in Ve values of BTU-HSA complex can be explained by the strong interactions between HSA molecules and the BTU.

The elution volumes of defect peaks increase from 110 to 114 ml when increasing the amount of injected HSA (BTU defect with respect to eluent composition). The shift observed in Ve can be attributed to adsorption mechanism of BTU on the gel rather than to a pure size exclusion effect. In fact, this behavior is quite common in gel filtration chromatography when binary equilibrating solutions are used (15,16). In our particular case, the adsorption of BTU on the gel was measured at 37°C, and a reversible adsorption was found. Due to this fact, Hummel and Dreyer's gel filtration method was compared with the method of equilibrium dialysis. The results of these experiments do not agree as shown in Figure 2.

Furthermore, when the BTU-HSA complex was dialysed in a phosphate buffer, a reversible binding of BTU to HSA was found. This reversibility gives rise to a dynamic equilibrium in the co-

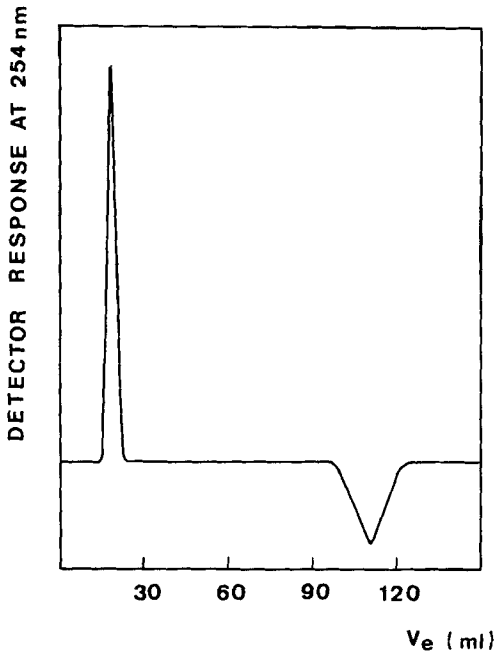

FIGURE 1. Chromatogram of binding of BTU to HSA. Mobile phase = 150 μM BTU in 0.067 M phosphate bufer, pH = 7.4. Sample = 1 ml of 72 μM HSA solved in the eluent. Flow rate = 0.62 ml/min. Chart speed = 2.5 cm/h.

lumn, between the BTU in the mobile phase and the BTU-HSA complex in the flow. Thus the concentration of BTU in the mobile phase is equivalent to the concentration of free BTU.

Since the BTU is adsorbed on the gel, the ligand concentration available for the protein in the mobile phase decreases. Therefore, to determine the free ligand concentration, L_f, the equation $L_f = L_t - L_{ad}$ should be used; L_{ad} being the ligand concentration adsorbed on the gel packed in to the column for an initial BTU concentration of L_t.

The amount of BTU that binds to HSA obtained from the chromatograms is described in Table 1, L_f being calculated according to the above equation.

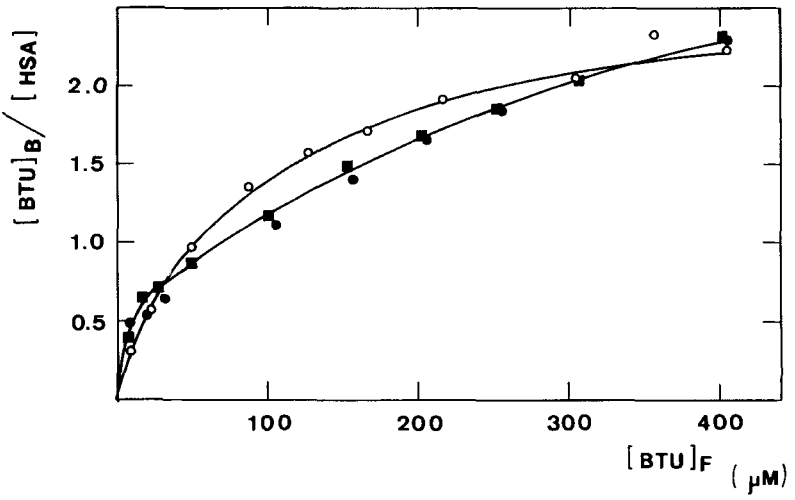

FIGURE 2. Binding of BTU to HSA by equilibrium dialysis method (o), and by gel filtration method (●, ■) for $[BTU]_F$ = BTU concentration in the eluent. [HSA] = 72 (■) and 145 μM (o,●).

TABLE 1

Binding of BTU to HSA from chromatograms at different eluent compositions.

L_t (μM)	BTU adsorbed (μmol/g gel)	L_{ad} (μM)	L_f (μM)	L_b* (μM)
20	0.07	1.84	18.16	78.70
30	0.17	4.18	25.82	90.78
50	0.35	8.87	41.13	150.31
100	0.85	20.60	79.40	158.38
150	1.29	32.31	117.69	197.54
250	2.23	55.78	194.22	240.99
300	2.70	67.51	232.49	256.29

* L_b = binding BTU concentration to 145 μM HSA

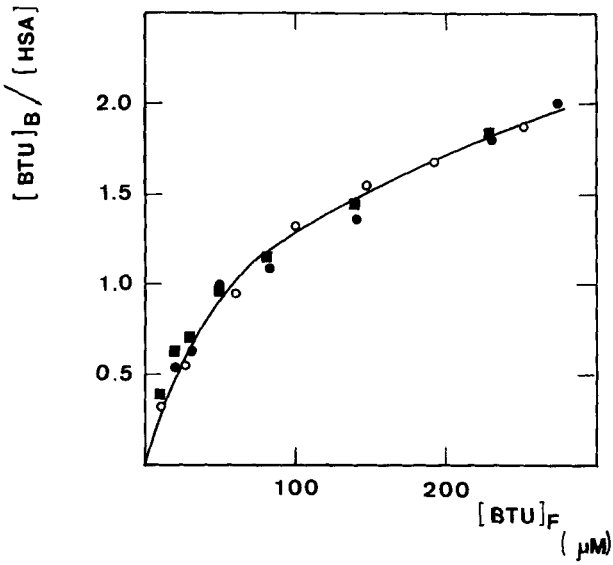

FIGURE 3. Binding of BTU to HSA by gel filtration method (●,■) for $[BTU]_F$ = free BTU concentration in mobile phase, and by equilibrium dialysis (o). [HSA] = 72 (■) and 145 μM (o,●).

As can be seen in Figure 3, these chromatographic data are in satisfactory agreement with those obtained by equilibrium dialysis.

The results obtained from both gel filtration and equilibrium dialysis are shown in a Scatchard plot (Figure 4) with two clear-cut classes of binding sites. One high affinity binding site and two low affinity sites. The apparent association constant, Ka, of the first class of binding sites is $5.7 \pm 0.05 \times 10^4\ M^{-1}$, while the Ka of the second class of binding sites is $7.9 \pm 0.10 \times 10^3\ M^{-1}$. Table 2 shows that the Ka values obtained with the two method are in satisfactory agreement.

The results obtained for the BTU association with HSA by Sephadex filtration, taking into account the amount of BTU adsorbed onto the gel, are in good agreement with those obtained by

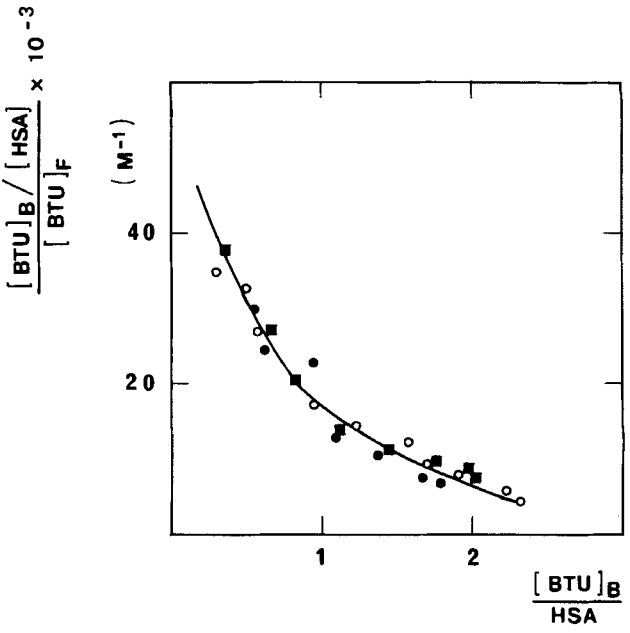

FIGURE 4. Scatchard plot for the binding of BTU to HSA. (o) Equilibrium dialysis method; (●, ■) gel filtration method ($[BTU]_F$ = free BTU concentration in mobile phase).

TABLE 2

Comparison of the Constants Characterizing the two clases of Binding Sites Obtained for the Binding of BTU to HSA by the Different methods used.

METHOD	$K_{1(M^{-1})}$	$K_{2(M^{-1})}$
Gel filtration without correcting L_f ($L_f=L_t$)	4.5×10^4	4.4×10^3
Gel filtration correcting L_f ($L_f=L_t-L_{ad}$)	5.7×10^4	7.8×10^3
Equilibrium dialysis	5.7×10^4	7.9×10^3

equilibrium dialysis while the shape of the curve for BTU-HSA binding without taking into account this BTU adsorption differs from that obtained by equilibrium dialysis (Figure 1). This corroborates the need to correct the chromatographic data, as we have described above, and makes evident the influence of the adsorption phenomenon on the calculation of the binding parameters, by gel filtration, when the ligand is adsorbed on the gel in the column.

We conclude, therefore, that if the free ligand concentration is correctly determined, the adsorption of ligand onto a gel does not interfere with the determination of binding parameters by Hummel and Dreyer's method.

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