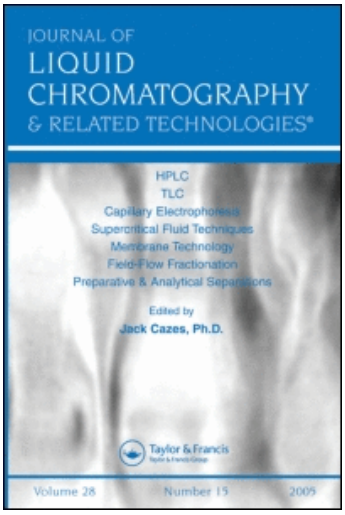

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DETERMINATION OF TRYPTOPHAN-HUMAN SERUM ALBUMIN BINDING FROM RETENTION DATA AND SEPARATION OF TRYPTOPHAN ENANTIOMER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention volume of a ligand, injected onto a size exclusion chromatographic column and eluted by a macromolecular complexing solution, is theoretically analysed for evaluating the binding between the solute and the macromolecule. The binding of D- and L- tryptophan to human serum albumin is given as an example, and the separation of these enantiomers is then achieved

INTRODUCTION

Recent studies have shown that the use of a complexing agent in the chromatographic mobile phase allows the separation of various compounds by liquid chromatography, as a result of selective formation of complexes between the solutes and the eluent. Several unsaturated and heterocyclic components have been separated by Ag^+ complexation (1) ; and some prostaglandins have been separated by cyclodextrin complexation (2). This latter work led to the determination of stability constants for drug-cyclodextrin

complexes (3). Sulfa drugs (4) and aminoacids (5), have been separated with mobiles phases containing metal chelate additives.

We reported, previously, the use of high performance liquid chromatography (H.P.L.C.) for determining drug-human serum albumin (HSA) binding parameters, using the measurement of the mean number of moles of ligand bound per macromolecule (6,7). The present work describes another way for H.P.L.C. determination of the binding of ligands to HSA : it involves measurement of the retention volume of the ligand injected onto a size exclusion chromatographic column and eluted by an albumin solution. The pore size of the support is choosen to exclude the albumin molecule and for increased ligand retention. The difference in D- and L-tryptophan complexation by HSA is then used to separate them from a racemic mixture.

THEORETICAL PART

When a ligand A binds reversibly to a macromolecule P by successive equilibria leading to the formation of PA, PA_2, ...PA_n complexes, the mean number of moles of ligand bound per mole of macromolecule, $\bar{r}$, is, according to Klotz (8), equal to :

$$\bar{r} = \frac{(A_{bound})}{(P_{total})} = \sum_{i=I}^{m} \frac{n_i K_i (A_{free})}{1 + K_i (A_{free})} \qquad \text{(Eq. 1)}$$

where (A_{bound}), (P_{total}), and (A_{free}) represent respectively, the concentrations of bound ligand, total macromolecule, and free ligand ; the number, m, represents the number of classes of indepen-

dent binding sites on the macromolecule such that each class, i, has n_i sites with binding affinity K_i. If we define α as the ratio of the concentrations of bound ligand to total ligand, at equilibrium, it is equal to :

$$\alpha = \frac{(A_{bound})}{(A_{total})} = \frac{\bar{r}\,(P_{total})}{(A_{total})} \qquad \text{(Eq. 2)}$$

The retention of a solute on a chromatographic column is a function of its affinities for both the stationary and mobile phases, and its elution volume, V_R, is given by the equation :

$$V_R = V_o + Kv_f$$

where V_o represents the elution volume of a solute that is not retained by the stationary phase, v_f is the volume of the stationary phase and K is the distribution coefficient of the retained solute between stationary and mobile phases.

If we consider the elution of a small molecule, A, on a size exclusion column, eluted by a solution of the macromolecule, P, which can complex A, leading to the formation of $PA, \ldots PA_n$ complexes, as described above, and if P is totally excluded from the stationary phase due to its great size, the complexes, PA_i, will also be excluded ; only the free solute, A, will then enter into the stationary phase. The retention volume of such a solute is equal to :

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

with K' being the distribution coefficient of the solute in the presence of the complexing macromolecule in the eluent. If K and V_R represent, respectively, the distribution coefficient and the

retention volume of the solute, on the same column, in absence of complexing agent, then :

$$K' = K(1 - \alpha)$$

since $(1 - \alpha)$ represents the ratio between the concentrations of free and total ligand respectively (from eq. 2). Consequently :

$$\frac{V'_R - V_o}{V_R - V_o} = \frac{K'}{K} = 1 - \alpha \qquad \text{(Eq. 3)}$$

Replacing α by its value from equation (2)

$$\frac{V'_R - V_o}{V_R - V_o} = 1 - \bar{r} \cdot \frac{(P_{total})}{(A_{total})} = \frac{1}{1 + \bar{r} \cdot \frac{(P_{total})}{(A_{free})}}$$

Thus :

$$\frac{V'_R - V_o}{V_R - V_o} = \frac{1}{1 + \sum_{i=1}^{m} \frac{n_i K_i (P_{total})}{1 + K_i (A_{free})}} \qquad \text{(Eq. 4)}$$

If the amount of the solute, A, injected onto the column is sufficiently low for considering the quantity $[K_i(A_{free}) + 1]$ as equal to 1, the equation (4) becomes :

$$\frac{V'_R - V_o}{V_R - V_o} = \frac{1}{1 + \sum_{i=1}^{m} n_i K_i (P_{total})} \qquad \text{(Eq. 5).}$$

So, for a given solute, V'_R is a hyperbolic function of the concentration of the macromolecule in the mobile phase (Eq. 5). The term $\sum_{i=1}^{m} n_i K_i$, representing the binding of the solute to

the macromolecule, is easily calculated from V'_R and V_R values. It is possible to set free of the V_R value (measured in absence of the macromolecule) by measuring the retention volumes V'_{R1} and V'_{R2} of the solute, for two macromolecule concentrations (P_1) and (P_2) respectively in the eluent. Then :

$$\frac{V'_{R1} - V_o}{V'_{R2} - V_o} = B = \frac{1 + \sum_{i=1}^{m} n_i K_i (P_2)}{1 + \sum_{i=1}^{m} n_i K_i (P_1)}$$

and

$$\sum_{i=1}^{m} n_i K_i = \frac{B - 1}{(P_2) - B(P_1)} \qquad \text{(Eq. 6)}$$

When the amount of the solute, A, injected onto the column is increased, the value of its free concentration in the column is enhanced, and its retention volume increases, as shown by the equation 4.

EXPERIMENTAL

Chromatographic Equipment : A 6000A pump, and U6K injector and a 440 UV detector, all from Waters Associates (Milford, Mass., U.S.A.), were used for all the experiments. The monitoring wavelength was 280 nm.

25 µl samples were injected onto a 15 cm length, 4.7 mm i.d., stainless steel column, filled by a slurry-packing technique with Lichrosorb Diol support, (Merck, Darmstadt, West Germany), 10 µm

particle diameter. This column allowed HSA to elute at the void volume of the column (1.48 ml) and D- and L-tryptophan to be retained longer (depending of the eluting solution). The column was thermoregulated at 20°C. The flow rate was 0.4 ml/mn.

Materials : D- and L-tryptophan were obtained from PROLABO (Paris, France). HSA was from SIGMA, (St Louis, MO, U.S.A.), as essentially fatty acid free albumin. All products were dissolved in a 0.067 M phosphate buffer, PH 7.4.

RESULTS AND DISCUSSION

Figure 1 represents the variation of D- and L-tryptophan retention volumes, as a function of the amount of the enantiomer in the sample, for three different albumin concentrations in the mobile phase. We notice first that D-tryptophan is not affected by the presence of HSA in the eluent ; its retention volume is constant and equal to 2.8 ml. However, L-tryptophan is eluted sooner with increasing concentrations of HSA, and its retention volume, equal to 2.8 ml for the more concentrated samples, decreases and reaches a limiting value when the concentration of L-tryptophan in the sample decreases. We have reported in Figure 2 the plot of these lowest values of L-tryptophan retention volumes as a function of the corresponding HSA concentrations : it agrees with the hyperbolic variation described by Equation 5.

The different chromatographic behaviours of D- and L-tryptophan enantiomers, observed in presence of HSA, agrees with

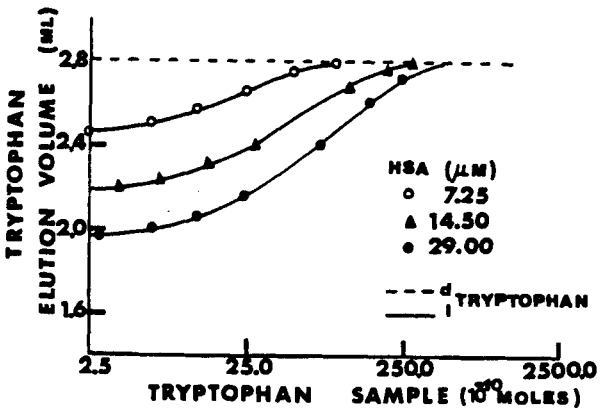

FIGURE 1

Variation of the retention volumes of L- and D-tryptophan with the concentration of the enantiomer in the sample, for different HSA concentrations in the mobile phase. Sample conditions : 25 μl of L- or D-tryptophan solutions in the eluent.

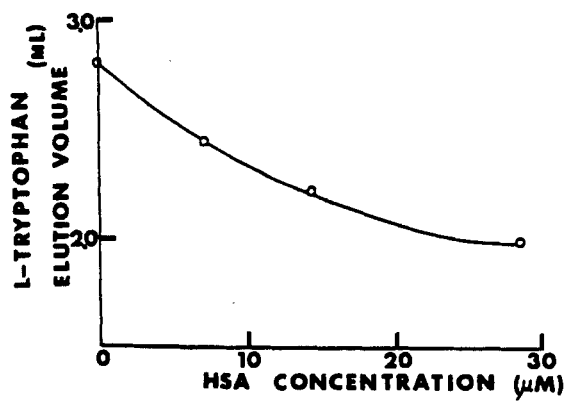

FIGURE 2

Plot of the lower limit retention volumes of L-tryptophan (obtained from Figure 1) as a function of the corresponding HSA concentrations in the mobile phase.

the difference of their affinities for HSA as measured by the equilibrium dialysis method (9) and gel filtration experiments (10) ; D- tryptophan is reported to bind to HSA about 100 fold less than the L-enantiomer does (10).

Table 1 represents the $\sum_{i=1}^{m} n_i K_i$ values obtained for L-tryptophan - HSA binding, at 20°C, from equations 5 and 6 respectively. The L-tryptophan retention volumes used for these calculations and reported in the table are the limit values obtained from Figure 1.

The $\Sigma n_i K_i$ values found are in agreement with each other. We have used selective affinity of HSA for L-tryptophan, described above, to separate the D- and L-enantiomers from a tryptophan racemic mixture. The chromatograms represented in Figure 3 show that the separation of the enantiomers increases as the HSA concentration in the mobile phase is increased and, for a given HSA concentration, when the amount of the sample of racemic

TABLE 1

L tryptophan - HSA Binding

P (M)	V_o (ml)	V'_R (ml)	$\Sigma n_i K_i$ from eq. 5 ($l.mole^{-1}$)	$\Sigma n_i K_i$ from eq. 6 ($l.mole^{-1}$)
0	1.48	2.80		
7.25	1.48	2.44	51.200	67.900
14.50	1.48	2.20	57.500	53.700
29.0	1.48	1.98	56.000	

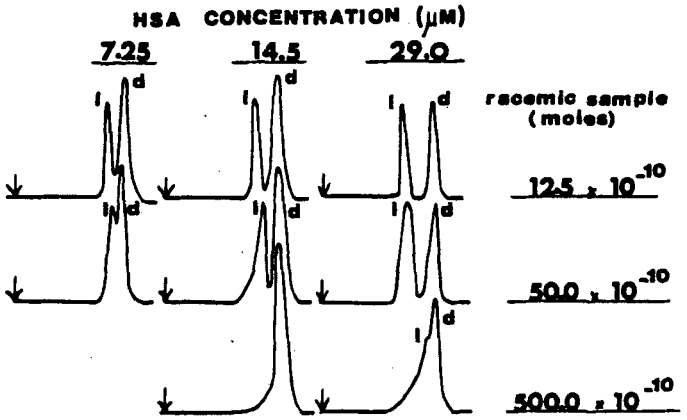

FIGURE 3

Chromatograms obtained for various samples of racemic tryptophan with different HSA concentrations in the mobile phase. Conditions as given in the experimental part.

tryptophan decreases. These D and L enantiomers have been resolved previously by affinity chromatography (11) on a bovine serum albumin-agarose column. This method of separation is also based upon the different binding of the optical isomers to the albumin molecule, but the advantage of the method we propose is that it does not necessite the preparation of an albumin-bonded chromatographic support, the HSA being, simply, dissolved in the phosphate buffer mobile phase.

CONCLUSION

We have described, in this report, a determination of the whole affinity of a ligand for a macromolecule from its chromatographic retention data in mobile phases containing a macromolecule. The behaviour of L-tryptophan in HSA mobile phases is

in accordance with the theoretical considerations, and this method may be used to separate small compounds exhibiting different affinities for the same polymer.

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