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High-performance liquid chromatographic determination of the binding of ceftriaxone to human serum albumin solution and albumin from diluted human serum

J. MOHLER*, A. MEULEMANS and M. VULPILLAT

Laboratoire de Biophysique, Faculté de Medecine Xavier Bichat, 16 Rue Henri Huchard, 75018 Paris (France)

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SUMMARY

The binding of ceftriaxone to human serum albumin has been studied by high-performance liquid chromatography. The gel permeation method of Hummel and Dreyer was used. Ceftriaxone was tested with two sources of albumin (aqueous solution and diluted serum). After internal calibration the binding parameters were determined for each albumin, and results compared. These data are in agreement with those from classical methods for the determination of protein binding of ceftriaxone.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been used for the measurement of ligand–protein binding mostly with the Hummel and Dreyer method [1,2]. This is an equilibrium method with the ligand in the mobile phase. A protein solution is injected into the column and eluted by the ligand solution. The presence of ligand in the eluent maintains a dynamic equilibrium similar to that in living systems.

The chromatographic support is chosen to exclude the macromolecule and to retain the ligand. The protein complex elutes before the excess of ligand. Detection of compounds at 254 nm produces large peaks, therefore the concentrations are determined by area calculations.

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Sebille et al. [3] used HPLC for the first determination of the binding of warfarin to human serum albumin. Results from gel permeation HPLC with LiChrosorb Diol columns are in complete agreement with those from other techniques. More recently Sun et al. [4] reported the same determination with equivalent results. Sun and Wong [5] also studied the binding of tryptophan to human serum albumin (HSA).

Ceftriaxone is a new cephalosporin with acid properties (pK_a 3.2–2.4). It has been confirmed that such acidic drugs are strongly bound to proteins (85–95%). The binding parameters [number of sites (n) and association constant (K_a)] have been determined for ceftriaxone by many authors, with equilibrium dialysis or ultrafiltration methods [11–14]. Our purpose was to determine the binding parameters by HPLC. The binding of ceftriaxone was studied with a solution of HSA at a concentration of 1.1 g/l and with diluted serum (46 g of albumin per litre of serum diluted to 1:40). We compared the protein binding of ceftriaxone to two different sources of albumin in order to evaluate its modification when biological molecules are present with albumin in the serum. The results of these two protein-binding studies are compared.

EXPERIMENTAL

Materials

HSA and essentially fatty acid-free albumin were from Sigma (St. Louis, MO, U.S.A.). The plasma was from a pool of volunteers not taking drugs. Protides and albumin were quantified to allow adequate dilutions. The final concentration was 1.14 g/l, corresponding to 17 μM. Ceftriaxone was from Roche Laboratory (Neuilly, France). All products were dissolved in 0.050 M phosphate buffer (pH 7.4).

Equipment

A Waters Assoc. 6000A pump and a Waters Assoc. 440 UV detector were used for all the experiments. An automatic injector WISP 710A (Waters France) was used for the repetitive injections.

Size-exclusion column

A Sep-Protein column (25 cm×7.5 mm I.D.) from SFCC (Neuilly Plaisance, France) was packed with spherical silica (20 μm particle size and 300 Å pore size). The silica was lined with a hydrophobic polymer. Specific details on the surface are proprietary, but the column packing shows low adsorption and denaturation. The Sep-Protein column can separate proteins with molecular weights ranging from 10^3 to 10^5 daltons. The silica must be used with suitable buffers, at the same conditions of pH and temperature as other size-exclusion chromatography columns. The concentration of ceftriaxone and pro-

tein should not be too high, so as not to obstruct the support or saturate the UV absorbance.

Experimental conditions

Solutions were prepared according the technique described in detail by Sun and Wong [5], who studied the binding of tryptophan to HSA.

Mobile phase. The stock solution for the mobile phase was prepared by dissolving 30 mg of ceftriaxone in 1 l of buffer (0.050 *M* phosphate, pH 7.4) and corresponded to 54 μM ceftriaxone. It was diluted to several concentrations with buffer, to produce four mobile phases for HSA solution and eight mobile phases for diluted serum (Table I).

Sample. The sample was a mixture of protein, ceftriaxone and buffer solutions to a total volume of 1250 μl. The volume injected into the column was 100 μl. A calibration was necessary to know the amount of bound drug as a function of protein, and we opted for an internal calibration.

Internal calibration. This calibration was described by Hummel and Dreyer [1] and Sun and Wong [5]. For each mobile phase we injected five samples (A, B, C, D, E) with increasing amounts of ceftriaxone (5.4–32.4 $\cdot 10^2$ μmol) and a constant amount of protein (6.8 $\cdot 10^2$ μmol). The samples are listed in Table II.

Calculation

Protein binding is a reversible process, described by the law of mass action [6–10]:

TABLE I

PREPARATION OF THE MOBILE PHASES

Dilution	Ceftriaxone (54 μM) volume (ml)	Buffer volume (ml)	Ceftriaxone in 100-μl sample (μmol·10^2)
Albumin solution			
1 5	100	400	10.8
2.5	200	300	21.6
3:5	300	200	32.4
4:5	400	100	43.2
Albumin in serum			
1 10	100	900	5.4
2·10	200	800	10.8
3 10	300	700	16.2
4:10	400	600	21.6
5:10	500	500	27.0
6 10	600	400	32.4
7 10	700	300	37.8
8 10	800	200	43.2

$$[P]+[C]_F=\frac{K_a}{K_d}[PC] \quad (1)$$

where [P] is the free protein concentration, $[C]_F$ is the free drug concentration and [PC] is the protein–drug complex concentration.

At equilibrium K_a is the association constant

$$K_a=\frac{[PC]}{[P][C]_F} \quad (2)$$

If R is the relation between the molar concentration of bound drug and the molar concentration of protein:

$$R=\frac{[PC]}{[PC]+[P]} \quad (3)$$

and from eqn. 2,

$$[PC]=K_a[P][C]_F$$

then

$$R=\frac{K_a[P][C]_F}{K_a[P][C]_F+[P]}$$

Hence eqn. 2 becomes

$$R=\frac{K_a[C]_F}{K_a[C]_F+1} \quad (4)$$

This equation can be transformed by the Scatchard method into:

$$R+RK_a[C]_F=nK_a[C]_F \quad (5)$$

The graph of $R/[C]_F$ versus R is linear, with the y axis representing nK_a, the x axis representing n; K_a is the slope.

RESULTS

Fig. 1 shows a typical chromatogram of protein binding determined by HPLC. The first peak is the protein complex widely excluded and the second (negative or positive) peak corresponds to the ligand. The area of the second peak was measured with a planimeter or an integrator.

Internal calibration

Results of the internal calibration are presented in Figs. 2 and 3. The samples A–E (Table II) injected into the column showed chromatograms similar to that of Fig. 1. The area of the second peak was measured. For each mobile

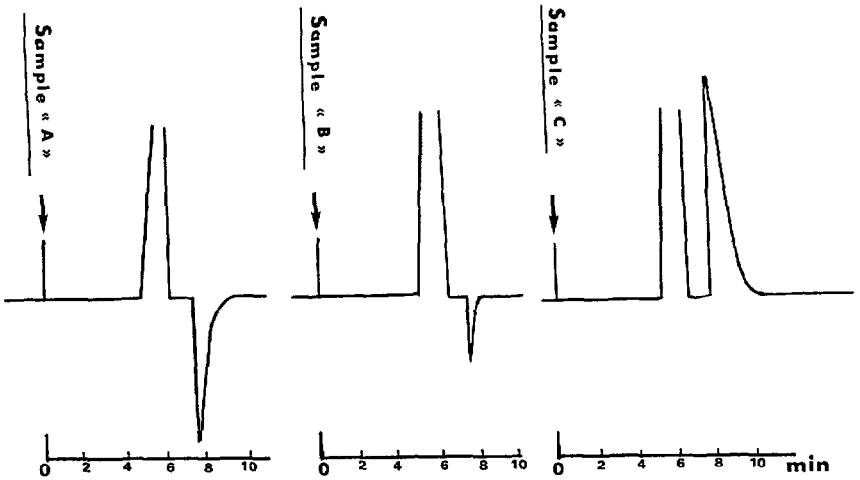

Fig. 1. Chromatograms of binding of ceftriaxone to HSA solution by size exclusion. Detection, UV at 254 nm, 0.05 a.u.f.s.; flow-rate, 1 ml/min; chart speed, 0.5 cm/min; mobile phase, 10.8 μM ceftriaxone in 0.05 M phosphate buffer (mobile phase 1.5 or 2·10). Sample A, 6.8 μM HSA and 5.4 μM ceftriaxone; sample B, 6.8 μM HSA and 10.8 μM ceftriaxone; sample C, 6.8 μM HSA and 16.2 μM ceftriaxone.

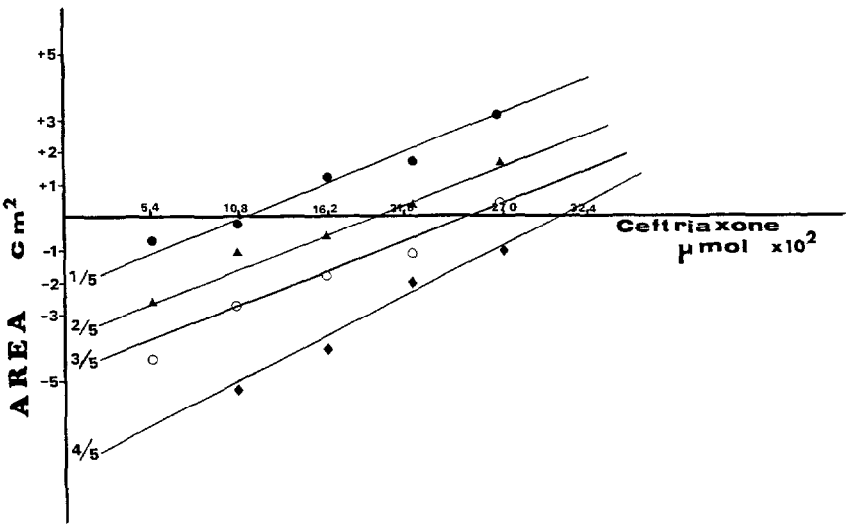

Fig. 2. Internal calibration for the determination of binding of ceftriaxone with serum albumin solution. Five samples (constant albumin and increasing drug amount) are in direct proportion to drug present in the mobile phase. The amount where area = 0 corresponds to bound drug.

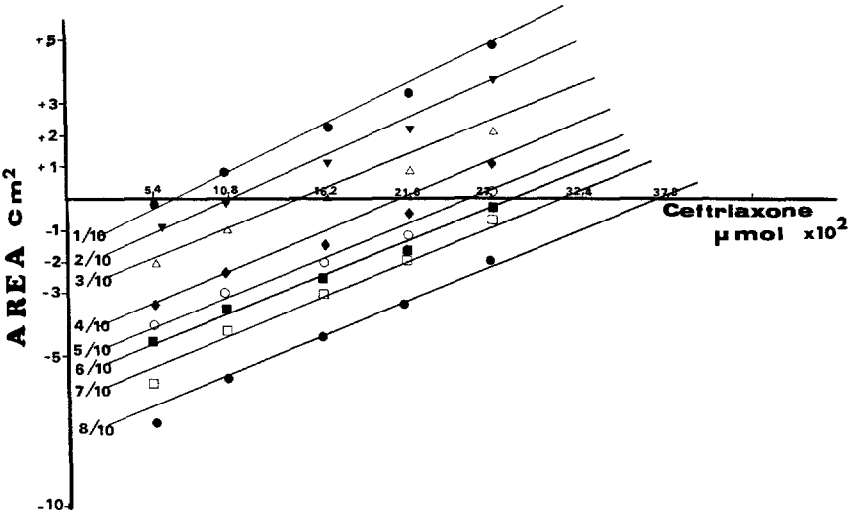

Fig. 3. Internal calibration for the determination of binding of ceftriaxone with diluted serum. Five samples (constant albumin and increasing drug amount) are in direct proportion to drug present in the mobile phase. The amount where area = 0 corresponds to bound drug.

TABLE II

PREPARATION OF SAMPLES

Sample	HSA volume (μl)	Ceftriaxone volume (μl)	Buffer volume (μl)	HSA concentration (μM)	Ceftriaxone concentration (μM)
A	500	125	625	6.8	5.4
B	500	250	500	6.8	10.8
C	500	375	375	6.8	16.2
D	500	500	250	6.8	21.2
E	500	750	-	6.8	32.4

phase (1:5 to 4:5 and 1:10 to 8:10, in Table I) the value of x corresponding to $y=0$ is the amount of bound drug. For example, in sample A (Table II) the concentrations of ceftriaxone and protein were 5.4 and 6.8 μM, respectively. Thus in 100 μl of the injected sample in the mobile phase (2:5 in Table I), the amount of bound ceftriaxone determined was $19.4 \cdot 10^2$ μmol. This procedure was repeated for all the mobile phases presented in Fig. 3.

A dynamic equilibrium exists between ceftriaxone and the ligand–protein complex in the mobile phase. The concentration of ceftriaxone in the mobile phase is equivalent to the concentration of free ceftriaxone, $[C]_F$.

From the concentration of protein injected into the column (Table II), the concentration of free drug (Table I) and the bound drug amount computed

from Figs. 2 and 3, we can determine the parameters of HSA–ceftriaxone binding.

Table III gives the results of bound ceftriaxone ($[C]_B$) found from the calibration, R, and $R/[C]_F$ for the Scatchard plot. The y axis represents nK_a and the x axis represents n (number of sites) (Fig. 4). First we found that the binding of ceftriaxone to albumin was the same with an albumin solution or with diluted serum at low concentration of compounds (see Table IV). This confirms the work of Brandebourger et al. [11], who tested many endogenous plasma compounds (gamma G, alpha G, haptoglobin, transferrin) for ceftriaxone binding. The protein binding of these endogenous compounds was always lower than 5% and did not affect the binding of ceftriaxone with albumin.

Thus we can state that ceftriaxone is bound by one binding site per albumin molecule ($n=0.99$). Brandebourger et al. [11] used the equilibrium dialysis method with a low concentration of human albumin (30 μM) and drug concentrations of 10–200 μM. They found the following binding parameters: $n=0.75+0.01$ and $K_a=71\,931+5883$. This confirms that there is only one binding site. The equilibrium dialysis method is the most classic procedure but errors in the osmotic effects and protein denaturation are frequent, as it requires two stages: the dialysis per se and the determination of each compartment by reversed-phase HPLC [12–17] or by scintillation counting [11,18]. The ultrafiltration system also presents limitations: several manipulations, a

TABLE III

INTERNAL CALIBRATION AND DATA FOR THE SCATCHARD PLOT

Dilution	$[C]_B$[a] (μmol) ($\times 10^2$)	R[b]	$R/[C]_F$[c]
Albumin solution			
1·5	10.8	0.61	5.675
2 5	19.4	0.74	3.425
3·5	26.0	0.79	2.444
4:5	31.2	0.82	1.898
Albumin in serum			
1.10	5.6	0.45	8.351
2 10	9.0	0.56	5 268
3 10	14.0	0.67	4.154
4:10	21.5	0.75	3.513
5 10	26.0	0.79	2.475
6:10	27.7	0.80	2.475
7:10	31.0	0.82	2.179
8·10	36.7	0.84	1.951

[a] $[C]_B$ = bound ceftriaxone; see Figs. 2 and 3.
[b] $R=[PC]/([PC]+[P])$; see eqn. 3.
[c] $[C]_F$ = free ceftriaxone; see Table I.

TABLE IV

PARAMETERS OF PROTEIN BINDING OF CEFTRIAXONE

	n	K_a
Ceftriaxone-HSA solution	0.99 ± 0.01	12 500 + 1700
Ceftriaxone-HSA from diluted serum	0.99 + 0.01	13 300 + 1100

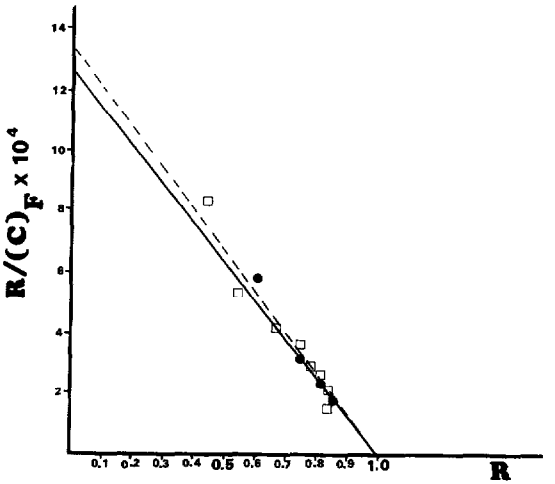

Fig. 4. Scatchard plot illustrating the binding of ceftriaxone to HSA: (●) HSA solution; (□) HSA from serum.

requirement for relatively large amounts of plasma and the use of HPLC [10,14,17].

The gel permeation is rapid since the results are obtained in the same analysis, in a few minutes. The main inconvenience is the limited amount of drug and protein that can be introduced into the column.

CONCLUSION

Gel permeation HPLC is quite suitable for the study of the protein binding of drugs. It has the advantages of being rapid, efficient and inexpensive, and it yields equivalent results to other techniques. The method is interesting in that it provides a rapid and inexpensive approach to the drug–albumin binding process and needs only small amounts of plasma. Progress in gel column technology will allow further studies on protein binding, as suggested by Sebille an Thuaud [8]. In the future, the application of this new method will be especially

useful for the study of physiological fluids such as cerebrospinal, pleural and extra-vascular fluids, in which albumin is present in low concentrations.

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