Biochemical Pharmacology, Vol. 37, No. 14, pp. 2807–2814, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

CEFAZOLIN SERUM PROTEIN BINDING AND ITS INHIBITION BY BILIRUBIN, FATTY ACIDS AND OTHER DRUGS

M. O. DECROIX,* R. ZINI,† J. C. CHAUMEIL* and J. P. TILLEMENT†

* Laboratoire de Pharmacie Galénique, U.E.R. Mécanismes d'Action des Médicaments et des Toxiques, 4 Avenue de l'Observatoire, F-75006 Paris Cedex, and † Service de Pharmacologie, Faculté de Médecine de Paris XII, 8 rue du Général Sarrail, F-94010 Creteil, France

(*Received* 9 *October* 1987; *accepted* 22 *February* 1988)

Abstract—This paper describes the protein binding of cefazolin to human serum and to human serum albumin (HSA) using equilibrium dialysis. The drug is exclusively bound to HSA with a moderate affinity, $K_a = 16600 \pm 1600\ M^{-1}$, and one saturable binding site, $n = 0.73 \pm 0.02$. Moreover cefazolin shows a dose-dependent binding leading a possible increase of the free fraction (when its total concentration increases). This antibiotic is displaced by free fatty acids (FFA) and bilirubin. Cefazolin binding to human serum and human serum albumin (HSA) was studied in presence of acidic drugs. At low concentrations clofibric acid and phenylbutazone both exhibiting high affinity for HSA displace strongly cefazolin. Valproic and salicylic acids, sulfamethoxazole, cefoperazone which have approximately the same affinity as cefazolin, must be used at higher concentrations to displace this antibiotic. A particular phenomenon was observed with cefazolin on HSA when associated with furosemide. A low concentration (5–25 μM) of this drug induces a positive cooperativity of binding between cefazolin and HSA. But at a molar ratio of furosemide to albumin greater than one, such cooperative interaction disappears and a competitive inhibition of cefazolin binding occurs. For all drugs studied, a competitive inhibition was found except for tryptophan. Finally, it is concluded that cefazolin shares the warfarin binding site on HSA.

Cefazolin is one of the most used first-generation cephalosporins. Only some data concerning the binding percentages of these cephalosporins to serum proteins were already described [1–3]. These antibiotics belong to the group of β-lactams and are weak acid drugs. As many acidic compounds, they bind to human serum albumin (HSA) on one single site [4]. Moreover, as for new cephalosporins such as ceftriaxone, a concentration-dependent protein binding within the range of therapeutic concentrations was reported [5–6].

Thus, the aim of the present study was to determine the binding characteristics of cefazolin to human serum and human serum albumin, the possible occurrence of a concentration-dependent binding and an eventual binding interaction between cefazolin and endogenous inhibitors was checked. Moreover the potential binding inhibition between cefazolin and salicylic acid, clofibric acid, valproic acid, sulfamethoxazole, phenylbutazone, furosemide, tolbutamide, warfarin, cefoperazone and tryptophan to HSA was checked. These experiments were carried out using the equilibrium dialysis method both in serum and isolated HSA solutions.

MATERIALS AND METHODS

Human serum. Human serum was obtained from a group of healthy volunteers (4 men, 3 women) ranging in age from 25 to 40 years. These patients were drug free and fasted before blood collection. Serum samples were pooled and stored at −30°. The total protein concentration was 65 g/l, and the HSA concentration was adjusted to 600 μM (40 g/l) with phosphate buffer (0.067 M). HSA concentration was estimated by the bromocresol green method. Free fatty acids (FFA) in pooled serum were extracted and measured by gas chromatography using the method of Sampson and Hensley [7]: myristic (12.3 μM), oleic (195.4 μM), palmitic (154.5 μM), palmitoleic (33.1 μM) and stearic (42.6 μM) acids.

The effect of bilirubin on cefazolin binding was studied with a pooled icteric serum from five patients whose total bilirubin concentration was 225 μM. Moreover, the same experiments were realized by adding bilirubin (MERCK, REF 24519) in the pooled normal serum to a final concentration of 225 μm. A HSA solution containing 600 μM palmitic acid (FFA/HSA molar ratio = 1) was used to study the interaction of cefazolin–palmitic acid on HSA.

Isolated proteins. HSA (Sigma A-1887, purity 99%, FFA/HSA = 0.04) was dissolved in the phosphate buffer 67 mM, pH 7.4, at a concentration of 25, 50 to 600 μM.

Alpha-1-acid glycoprotein (AAG, purity 99%, Behringwerke) was dissolved in the phosphate buffer 67 mM, pH 7.4, at a concentration of 25 μM.

γ-Globulins (IgG, Sigma HG II, 99% purity) were also dissolved in the same phosphate buffer at a concentration of 100 μM.

Lipoproteins (VLDL, LDL and HDL) were isolated by ultracentrifugation (Beckman L5-50 B, rotor 50 Ti) from pooled normolipidaemic human serum, according to the procedure described elsewhere [4].

Briefly, serum was diluted (1:1) in 0.9% NaCl and centrifuged at 200,000 *g* at 4° for 16 hr. Supernatant (VLDL) was collected and the remaining solution was adjusted to 1.063 density (*d*) with 3 M KBr ($d = 1.35$). Then, this solution was centrifuged at 200,000 *g* at 4° for 16 hr. The supernatant (LDL) was collected and the remaining solution was adjusted to $d = 1.210$ with 3 M KBr and centrifuged at 200,000 *g* at 4° for 40 hr. The supernatant (HDL) was collected. Each lipoprotein was purified by a second ultracentrifugation corresponding to its density for the same time.

The concentrations were 0.1, 1 and 10 μM for VLDL, LDL and HDL respectively using average molecular masses of 10^7, 3×10^6 and 3×10^5 Da for VLDL, LDL and HDL respectively.

Cephalosporins. Unlabeled and labeled cefazolin were kindly provided by Eli Lilly. The structure of ^{14}C labeled cefazolin (96.2 kBq/mg) and the labeled position ^{14}C cefazolin were shown (Fig. 1). The purity of the antibiotic was checked by thin-layer chromatography (Silica gel F-254, Merck) using the following solvent system: methanol: ammoniac (50:1). The radiochemical purity was higher than 96%. ^{14}C-cefazolin was used over a range of concentrations from 3 to 300 μM.

Interactions between cefazolin and other drugs. These studies were realized using both HSA, pooled serum and ^{14}C-cefazolin. Briefly, with a HSA sample, poor in FFA (Sigma, A-2187), different experiments were carried out using cefazolin alone or in the presence of different drugs. The HSA concentration was adjusted to 25 or 50 μM with phosphate buffer (67 mM). The same experiments were also carried out using human pooled serum at a total protein concentration of 65 g/l.

The drugs used were as follows: salicylic acid (Rhône-Poulenc), clofibric acid (ICI), valproic acid (Labaz), phenylbutazone (Ciba-Geigy), sulfamethoxazole (Roche), warfarin (Merrell), furosemide and tolbutamide (Hoechst) and L-tryptophan (Sigma T 0254). All competition binding studies were carried out by equilibrium dialysis.

Equilibrium dialysis. Drug binding to HSA was studied by equilibrium dialysis. The experiments were carried out at 37°, pH 7.4 in 67 mM phosphate buffer, under a constant stirring at 20 rpm (DIANORM®) for 3 hr sufficient to reach the equilibrium for all the compounds tested (Zini, personal data). No significant binding to the dialysis binding (Visking) and cell walls was observed (Zini, personal data). In addition, no significant variation in the volume and pH was noticed after dialysis. At the end of each experiment, concentrations in each compartment were measured by liquid scintillation counting (Packard Tricarb Liquid Scintillation Spectrometer CD 360).

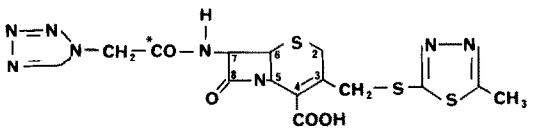

Fig. 1. Structure of ^{14}C labelled cefazolin.

Calculations. The binding percentage was determined from the concentration obtained at equilibrium:

$$\text{Drug binding}\,(\%) = B/(B + F) \times 100 \qquad (1)$$

At equilibrium, the bound (B) and free (F) concentrations of cefazolin were measured and binding parameters determined according to Zini *et al.* [8] and the curve B = f(F) was plotted. When the HSA binding was found to follow a saturable process, the association constant, K_a, the total concentration of binding sites in the protein solution, N, and the number of binding sites per protein molecule, n, were calculated according to the equation [8, 9]

$$B = \frac{NK_aF}{1 + K_aF} = \frac{nRK_aF}{1 + K_aF} \qquad (2)$$

where R denotes the concentration of the protein.

In the presence of a competitive binding inhibitor, the following equation was used

$$B = \frac{NK_aF}{1 + K_aF + K_II} \qquad (3)$$

where K_I and I denote the association constant and the free concentration of the inhibitor, respectively; K_a represents the association constant of cefazolin. Data obtained with an inhibitor were treated as follows: bound (B) versus free (F) ligand plots, obtained with and without different concentrations of inhibitor, were analyzed simultaneously assuming that either n or K_a values depended on the inhibitor concentration. The correct model was then chosen according to the best fit [9, 10].

The binding parameters were calculated using a non-linear least-squares method based on a Gauss–Newton algorithm [8, 9]. All data were analyzed with a uniform weighting where deviations observed are proportional to measured B and F values, i.e. all weights were set equal to one. The program was run on a Tektronix 4051 microcomputer. Finally two curves B = f(F) and B/F = f(B) were plotted.

RESULTS

Cefazolin binding to HSA and serum

The percentage of cefazolin bound to either HSA or serum remained constant until 200 μM, then decreased rapidly with the increase of cefazolin concentration. However, binding was higher to HSA than in serum for identical concentrations (Table 1).

Binding of cefazolin over the range of 3–260 μM was studied at 50 μM HSA. The results showed one saturable binding site ($n = 0.73 \pm 0.02$) with a moderate affinity ($K_a = 16{,}600 \pm 1600\,M^{-1}$) and the Scatchard plot revealed only one class of binding sites (Fig. 2).

Cefazolin binding to AAG, lipoproteins and γ-globulins

No binding occurred to these proteins.

Effect of FFA and bilirubin on cefazolin binding to HSA

The difference of cefazolin binding observed between human serum and HSA led to verification

Table 1. Cefazolin binding percentages to serum proteins, human serum albumin (HSA) and HSA in the presence of palmitic acid

Cefazolin (μM)	Serum	HSA (600 μM)	HSA (600 μM) +palmitic acid (600 μM)
10	78.7 ± 2.1	83.5 ± 1.1	81.0 ± 1.9
20	80.2 ± 3.0	83.5 ± 0.6	81.3 ± 0.7
40	80.8 ± 0.4	82.8 ± 1.9	80.5 ± 0.6
60	79.4 ± 1.0	82.8 ± 1.4	81.2 ± 0.3
80	78.9 ± 1.2	82.5 ± 1.2	80.4 ± 0.4
120	78.8 ± 0.5	81.0 ± 0.1	*74.7 ± 0.8
160	77.6 ± 0.3	79.1 ± 1.2	*72.8 ± 0.6
200	75.2 ± 0.5	76.8 ± 0.6	*71.2 ± 2.8
260	74.7 ± 0.1	75.5 ± 0.8	*68.1 ± 1.6
300	70.4 ± 0.2	70.2 ± 0.6	*65.4 ± 1.7

Each percentage represents the mean of three measurements with the standard deviation (M ± SD).
* $P < 0.01$ (Student test).

of the importance of FFA, namely palmitic acid (molar ratio = 1). The cefazolin binding to HSA was higher than that of HSA with palmitic acid (Table 1). These data were very similar to those obtained in serum, except for the high concentrations where palmitic acid showed effects more pronounced than those obtained with HSA alone (Table 1). So palmitic acid was able to decrease the cefazolin binding to HSA, as FFA present in serum did, but to a lesser extent. When bilirubin was present either in normal serum or in pooled icteric serum, the cefazolin binding decreased compared to that observed in pooled normal serum (Table 2). This decrease was much more important in pooled icteric serum (about 50%) than that observed in normal serum (about 7%) overloaded with the same concentration of bilirubin (Table 2).

Inhibition of cefazolin binding to HSA by other drugs

The affinity constant of cefazolin was lowered in the presence of all drugs except tryptophan. Table 3 shows that this decrease was proportional to the affinity and the concentration of the drugs studied. At low concentrations, clofibric acid (3 μM) and phenylbutazone (5 μM) induced an important decrease of cefazolin affinity ($K_a < 8500\ M^{-1}$). More important concentrations of sulfamethoxazole (50 μM), valproic acid (40 μM), salicylic acid (10 μM), and cefoperazone (40 μM) were required to observe a significant decrease of cefazolin affinity constant ($K_a < 9200\ M^{-1}$). So a low concentration of valproic acid (20 μM) or sulfamethoxazole (20 μM) did not show a variation of cefazolin affinity constant (Table 3).

Table 4 shows a particular phenomenon: with warfarin (10 μM), furosemide (5 and 25 μM) and tolbutamide (5 μM), a persistence or a light increase (statistically not significant, except for 25 μM furosemide) of cefazolin affinity was observed. The affinity constant then decreased rapidly with higher concentrations of these drugs. For example, the affinity constant became $3900\ M^{-1}$ with 100 μM of warfarin, $5400\ M^{-1}$ with 50 μM of tolbutamide and moreover no binding of cefazolin could be seen with 100 μM of furosemide (Figs 3 and 4).

Cefazolin binding to normal serum in the presence of drugs

At the higher saturable therapeutic concentration of cefazolin (250 μM), the percentage of cefazolin bound alone or in presence of tryptophan (50 μM), warfarin (10 μM), furosemide (1 μM) remained constant (75%) (Table 5). On the other hand, cefazolin was displaced by the other drugs inducing an important increase of free cefazolin from 25 to 100% particularly with sulfamethoxazole (800 μM) and phenylbutazone (446 μM). A lower increase of free

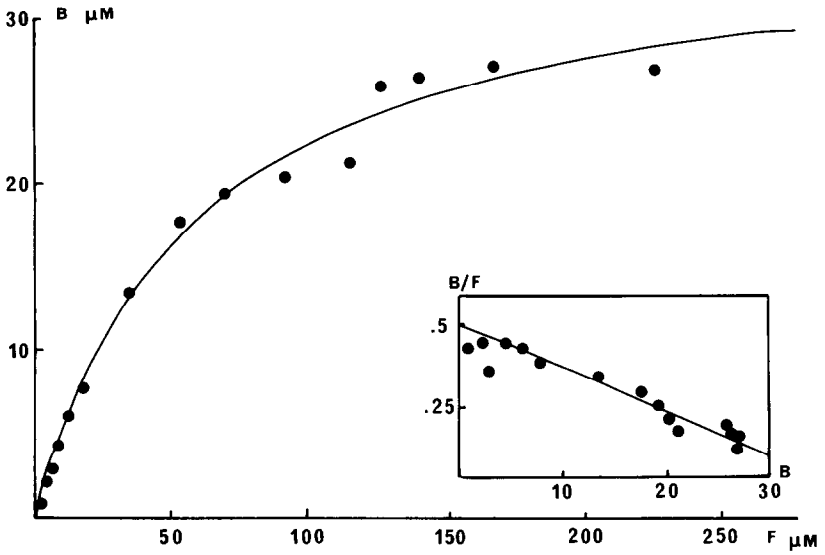

Fig. 2. Binding of cefazolin to HSA. The HSA concentration is 50 μM. B and F are respectively the concentration of bound and free cefazolin. Each point represents the mean ± SD of three determinations. The insert is the Scatchard plot.

Table 2. HSA Cefazolin binding percentages alone and in the presence of bilirubin

Cefazolin (μM)	Normal serum (HSA = 600 μM)	Icteric serum (HSA = 480 μM)	Normal serum +bilirubin (225 μM)
10	78.7 ± 2.1	*24.0 ± 4.6	*72.0 ± 2.0
40	80.8 ± 0.4	*19.7 ± 0.1	*72.0 ± 0.5
100	78.6 ± 0.7	*15.4 ± 1.6	*70.5 ± 0.5
200	75.2 ± 0.5	*16.2 ± 2.3	*67.0 ± 0.1
300	70.4 ± 0.2	*16.0 ± 0.7	*63.0 ± 1.0

Icteric serum contained 480 μM HSA and 225 μM bilirubin.
Each percentage represents the mean of three measurements with the standard deviation (M ± SD).
* $P < 0.01$ (Student test).

cefazolin, varying from 25% to 50%, was observed in the presence of salicylic acid (7200 μM), tolbutamide (200 μM), clofibric acid (630 μM) and valproic acid (600 μM).

DISCUSSION

Cefazolin binding to serum and HSA

Our data shows that HSA is the only protein able to bind cefazolin. Neither α_1-acid glycoprotein (AAG) nor lipoproteins and γ-globulins could bind cefazolin. Moreover the results show one saturable binding site ($n = 0.73 \pm 0.02$) with a moderate affinity ($K_a = 16{,}600 \pm 1600\ M^{-1}$) to HSA. These results are in accordance with those found for other cephalosporins [3–5] and other acidic drugs [11]. At cefazolin therapeutic concentrations and HSA physiological concentration, the percentage of cefazolin bound to serum remained constant until 200 μM of total cefazolin, then decreased rapidly when the total drug concentration increased (Table 1).

So cefazolin showed a binding dependent on its total concentration leading to an increase of the cefazolin-free fraction (about 76%) when the cefazolin total concentration was higher than 200 μM. This phenomenon allows comparison of the cefazolin binding to the third generation cephalosporin binding, particularly ceftriaxone [5, 6]. Ceftriaxone and cefazolin present the same pharmacokinetic profile where their apparent volume of distribution (V_d) is low and superposable to HSA's, i.e. 0.1 l/kg.

Cefotetan, cefonicid, cefoperazone, ceforanide [12–15] show also a low V_d ranging from 0.1 to 0.2 l/kg which increases when the total concentration of these drugs rises.

This saturable process is likely to be of importance in clinical and pharmacokinetic studies. The unbound fraction of the drug available will change extensively within the range of therapeutic concentrations, thus influencing the volume of distribution and the clearance of cefazolin which shows also a low extraction ratio.

Other drugs show also a binding dependent on their total concentration. These drugs are essentially acidic drugs such as salicylic acid, valproic acid,

Table 3. Cefazolin affinity constant (K_a) for HSA in the presence or absence of inhibitors

Drugs	Inhibitor concentration (μM)	K_a (M^{-1})
Cefazolin alone		16,600 ± 1600
Cefazolin +		
Clofibric acid	3	*8500 ± 2500
Valproic acid	20	13,000 ± 3000
	40	*11,500 ± 2600
Phenylbutazone	5	*6600 ± 970
Salicylic acid	10	*9200 ± 1400
Sulfamethoxazole	20	16,500 ± 3300
	50	*7500 ± 1000
Tryptophan	10	15,800 ± 1800
Cefoperazone	40	*4100 ± 1600

Cefazolin concentrations varied from 3 to 300 μM; HSA was 25 μM.
Each value represents the mean of three measurements with the standard deviation (M ± SD).
* $P < 0.01$ (Fisher's test).

Table 4. Cefazolin affinity constant (K_a) for HSA alone and in the presence of inhibitors

Drugs	Inhibitor concentration (μM)	K_a (M^{-1})
Cefazolin alone		16,600 ± 1600
Cefazolin +		
Warfarin	5	*12,700 ± 2400
	10	14,000 ± 4000
	25	17,000 ± 3200
	100	3900 ± 580
Furosemide	5	21,300 ± 2600
	25	*26,200 ± 10,600
	50	*5200 ± 2500
	100	No binding
Tolbutamide	2	*11,600 ± 1800
	5	13,400 ± 4200
	50	*9200 ± 2700

Cefazolin concentrations varied from 3 to 300 μM; HSA was 50 μM.
Each value represents the mean of three measurements with the standard deviation (M ± SD).
* $P < 0.01$ (Fisher's test).

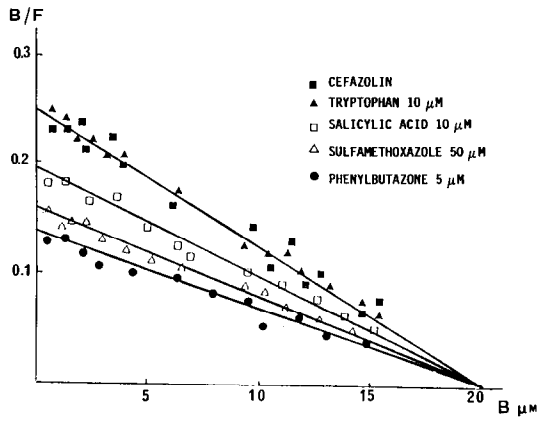

Fig. 3. Scatchard plot showing the binding of cefazolin to HSA (25 μM) in the absence or presence of inhibitors.

phenylbutazone and sulfonamides, which bind only to HSA.

On the other hand, basic drugs such as lidocaine, quinidine, disopyramide, propisomide [16–19] and erythromycin [20] bind essentially to AAG and show non-linear pharmacokinetics due to binding dependency.

Interaction between cefazolin and FFA to HSA

Our results show that cefazolin binding percentage to HSA is higher than that obtained with HSA containing palmitic acid and human serum. This phenomenon can be explained by the ability of palmitic acid and FFA to displace cefazolin from its HSA binding site.

As reported by some authors [21–24], HSA would possess five binding sites with only two for endogenous specific ligands (FFA and bilirubin) and three for the drugs showing a saturable binding (warfarin,

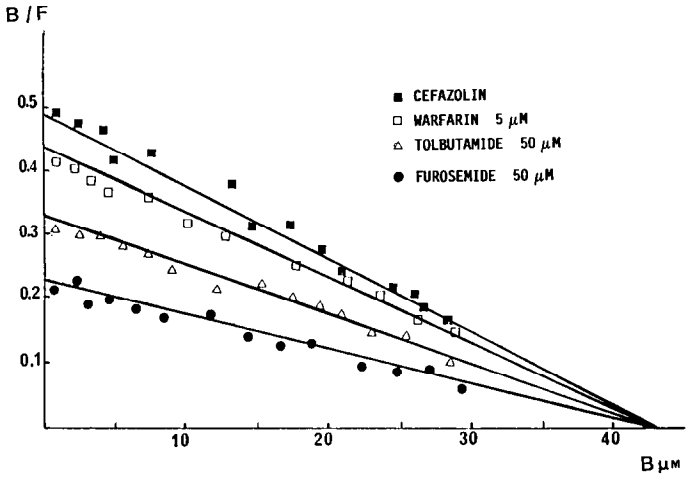

Fig. 4. Scatchard plot showing the binding of cefazolin to HSA (50 μM) in the absence or presence of inhibitors.

Table 5. Binding (%) and free cefazolin concentration in serum alone and in presence of inhibitor therapeutic concentrations

Inhibitor (μM)	Free cefazolin (μM)	Binding percentage
Cefazolin alone 250 μM	110.7 ± 0.9	75.0 ± 0.5
Cefazolin +		
Clofibric acid 630 μM	165.1 ± 19.2*	59.3 ± 2.8*
Valproic acid 600 μM	153.9 ± 5.1*	62.4 ± 1.1*
Phenylbutazone 446 μM	216.6 ± 8.1*	57.9 ± 3.3*
Salicylic acid 7200 μM	137.5 ± 7.9*	40.3 ± 1.8*
Sulfamethoxazole 800 μM	198.1 ± 7.6*	53.9 ± 1.6*
Tryptophan 50 μM	114.5 ± 2.4	74.05 ± 0.5
Warfarin 10 μM	113.2 ± 3.8	75.04 ± 0.6
Furosemide 1 μM	109.3 ± 0.6	75.2 ± 0.5
Tolbutamide 200 μM	145.7 ± 14.7*	62.8 ± 1.9*

The HSA concentration in serum was 600 μM.
Each value represents the mean ± SD of five determinations.
* $P < 0.01$ (Fisher's test).

diazepam and digitoxin). FFA with long chains ($>C_{10}$) such as lauric acid, myristic acid, palmitic acid, stearic acid would own two sites with high affinity [23, 25] independent of warfarin, diazepam and digitoxin sites. However, binding of FFA to their own sites would induce a structural change of HSA modifying the binding of other drugs [26].

Our results would seem to verify this hypothesis. When palmitic acid was added to HSA, its binding would lead to a structural change of HSA and it would result in a decrease of the cefazolin binding to HSA.

Interaction between cefazolin and bilirubin to HSA

Bilirubin is known to have one high affinity binding site $K = 10^8\,M^{-1}$ [27] and one or two sites with a moderate affinity on HSA [28]. The first is localized between warfarin and diazepam sites and the second would be identical to warfarin's [29–33].

In our data, bilirubin/HSA ratio is ≤ 0.5. So HSA-bilirubin binding is non-saturable, bilirubin occupying only its first site (99.9%). The second site, identical to the warfarin site, is not occupied at this bilirubin concentration. The decreased cefazolin binding could be explained by an allosteric effect (HSA structural change) induced by the bilirubin binding to its high affinity site. Moreover this effect is accentuated by the HSA low concentration (480 μM) in icteric serum (hypoalbuminemia). So for all these reasons, in the pathological serum, this inhibition seems to be non-competitive. The important decrease of cefazolin binding percentage observed would be accentuated both by the high bilirubin concentration, the hypoalbuminemia, and perhaps the presence of unknown endogenous inhibitors as demonstrated by Takeda *et al.* [34] in uremic serum. Moreover the albumin structure may be also changed in few pathological states.

Interaction between cefazolin and other drugs to HSA

Cefazolin was characterized by a moderate affinity ($K_a = 16{,}600\,M^{-1}$) and showed only one binding site to HSA.

The choice of inhibitors was determined respectively by their affinity constants and their binding sites. These drugs have often one saturable binding site except tolbutamide, valproic acid and sulfamethoxazole where $n = 2$. Their affinity constant was higher than cefazolin's, varying from $24{,}600\,M^{-1}$ for cefoperazone to $330{,}000\,M^{-1}$ for clofibric acid. Only sulfamethoxazole possesses a very low affinity ($K_a = 5000\,M^{-1}$; Zini, personal data).

Concerning clofibric acid and phenylbutazone, both presenting a high affinity ($>200{,}000\,M^{-1}$), an important decrease of cefazolin affinity was observed at very low concentrations of phenylbutazone and clofibric acid (Table 3).

But with the drugs having a moderate affinity such as sulfamethoxazole and valproic acid, no variation of cefazolin affinity was noticed at low concentrations (20 μM) but a very significant decrease of this affinity was found at upper concentrations, i.e. 50 μM. Cefoperazone, belonging to the same chemical and pharmacological class as cefazolin, possesses a higher affinity than that of cefazolin [4, 14]. This antibiotic competitively displaces cefazolin, leading to the hypothesis that cefazolin and cefoperazone share the same binding site on HSA. Finally, clofibric acid and phenylbutazone at low concentrations, strongly displace cefazolin because their affinities for HSA are very high.

On the other hand, valproic acid, sulfamethoxazole, salicylic acid and cefoperazone, having equivalent affinity to that of cefazolin, must be used at higher concentrations to displace cefazolin from HSA. For all these inhibitions, a decrease of cefazolin affinity was observed, but its number of binding sites was unchanged. So these drugs induced a competitive inhibition of cefazolin binding to HSA except tryptophan for which no change of the affinity and the number of binding sites of cefazolin was observed (Fig. 3 and Table 3). Table 4 shows an increase of cefazolin affinity at low concentrations of furosemide (5 and 25 μM), while a decrease of this cefazolin affinity was noticed for the high concentrations (≥ 50 μM) of warfarin, furosemide and tolbutamide. Although the observed increase of cefazolin affinity in presence of 5 μM furosemide was not statistically significant, this apparent affinity became statistically significant ($P < 0.01$) with 25 μM of furosemide. These results strongly suggest that the interaction between cefazolin and furosemide could be a positive cooperativity. However, this phenomenon was not seen when higher concentrations of furosemide were used (Fig. 4 and Table 4).

Chakrabarti [35] has already noticed this phenomenon with warfarin in the presence of oleate ion on HSA. A low molar ratio of oleate and HSA induced a positive cooperativity of binding between warfarin and HSA.

With a high molar ratio, this cooperative interaction disappeared and then a simple competitive inhibition was observed. Owing to furosemide, our results seem to follow the same phenomenon. These findings would suggest a conformational change in the albumin molecule at low concentrations of inhibitors, such as warfarin, furosemide, tolbutamide. On the other hand at a molar ratio of inhibitors to albumin greater than one, such a cooperative interaction disappeared and a competitive inhibition of cefazolin binding was observed (Fig. 4).

On the other hand, warfarin and tolbutamide at low concentration showed a trend towards a positive cooperativity between the cefazolin binding and these inhibitors to HSA. However, there was absolutely no evidence, statistically significant, to show definitely a positive cooperativity between cefazolin and warfarin or tolbutamide because the cefazolin affinity was perhaps too low. Scatchard plots (Figs 3 and 4) show that salicylic acid, sulfamethoxazole, phenylbutazone, furosemide, tolbutamide, warfarin were competitive inhibitors; for tryptophan no inhibition was found. The cefazolin affinity was decreased for inhibitors sharing the same site on HSA. All these inhibitors belong to group I [21, 22, 24] and bind to warfarin site. So it can be concluded that cefazolin was bound to the warfarin site. On the other hand, tryptophan [22] belongs to group II, i.e. diazepam site [22], and did not displace cefazolin.

At therapeutic concentrations, the percentage of bound cefazolin to HSA in the presence of trypto-

phan, warfarin and furosemide remained constant, leading to an unchanged cefazolin-free fraction.

In return the association of cefazolin with sulfamethoxazole or phenylbutazone could lead to an adjustment of dosage. However, a twofold increase in free fraction of cefazolin does not mean that the free concentration of cefazolin increases twofold because the clearance of cefazolin can be also increased. On the other hand, when a chronic treatment is set up with two drugs (for example cefazolin and phenylbutazone), an equilibrium takes place between the free and bound fractions of the two drugs, although a displacement of bound cefazolin is temporarily possible for the first administration of the second drug. So, during a therapeutic treatment with a drug only, it is important to distinguish the consequences of the administration of a second drug inhibiting the binding of the first one, whether the administration is a single dose or a multiple dose. Finally the presence of salicylic acid, tolbutamide, clofibric acid and valproic acid at very high therapeutic concentrations increased the free cefazolin concentration *in vitro* (Table 5).

In conclusion, cefazolin shows a binding dependent on its total concentration leading to a possible increase of its plasma-free fraction when high blood concentrations are reached. This increase in free fraction was also observed with cefazolin and other drugs occupying the same binding site on HSA.

Acknowledgements—We thank Dr Salord from Eli Lilly Laboratories for his generous gift of both labeled and unlabeled cefazolin.

REFERENCES

1. Nightingale CH, Greene DS and Quintilliani R, Pharmacokinetics and clinical use of cephalosporin antibiotics. *J Pharm Sci* **64**: 1899–1927, 1975.
2. Rolinson GN and Sutherland R, The binding of antibiotics to serum proteins. *Br J Pharmacol* **25**: 638–644, 1965.
3. Decroix MO, Chaumeil JC and Flouvat B, Binding of cefalotin to human serum albumin. *Eur J Drug Metab Pharmacokin* **9**: 191–194, 1984.
4. Watanabe Y, Hayashi T, Takada R, Yasuda T, Saikawa I and Shimizu K, Studies on protein binding of antibiotics. I Effect of cefazolin on protein binding and pharmacokinetic of cefoperazone. *J Antibiot* **33**: 625–635, 1980.
5. Stoeckel K, McNamara PJ, Brandt R, Plozzanottebrock H and Ziegler WH, Effects of concentration-dependent plasma protein binding on ceftriaxone kinetics. *Clin Pharmacol Ther* **29**: 650–657, 1981.
6. McNamara PJ, Gibaldi M and Stoeckel K, Volume of distribution terms for a drug ceftriaxone. Exhibiting concentration-dependent protein binding. I Theoretical considerations. *Eur J Clin Pharmacol* **25**: 399–405, 1983.
7. Sampson D and Hensley WJ, A rapid gas chromatographic method for the quantitation of underivatized in individual free fatty acids in plasma. *Clin Chim Acta* **61**: 1–8, 1975.
8. Zini R, D'Athis P, Barre J and Tillement JP, Binding of indomethacin to human serum albumin, its non displacement by various agents, influence of free fatty acids and the unexpected effect of warfarin binding. *Biochem Pharmacol* **28**: 2661–2665, 1979.
9. Morin D, Zini R, Ledewyn S, Colonna JP, Czajka M and Tillement JP, Binedaline binding to plasma proteins and red blood cells in humans. *J Pharm Sci* **74**: 727–730, 1985.
10. Urien S, Albengres E, Zini R and Tillement JP, Evidence for binding of certain acidic drugs to 1 acid-glycoprotein. *Biochem Pharmacol* **31**: 3687–3689, 1982.
11. Tillement JP, Zini R, D'Athis P and Vassent G, Binding of acidic drugs to human albumin: Theoretical and practical estimation of fundamental parameters. *Eur J Clin Pharmacol* **7**: 307–313, 1974.
12. Nakagawa K, Koyama M, Tachibana A, Komiya M, Kikuchi Y and Yano K, Pharmacokinetics of cefotetan (YM 09330) in humans. *Antimicrob Agent Chemother* **22**: 935–941, 1982.
13. Barriere SL, Hatheway GL, Gambertoglio JG, Lin E and Conte JE, Pharmacokinetics of cefonicid, a new broad-spectrum cephalosporin. *Antimicrob Agent Chemother* **17**: 935–938, 1982.
14. Garzone P, Lyon J and Yu VL, Third-generation and investigational cephalosporins. I—Structure activity relationships and pharmacokinetic review. *Drug Intell Clin Pharm* **17**: 507–515, 1983.
15. Lee FH, Pfeffer M, Van Harken R, Smyth RD and Hottendorf GH, Comparative pharmacokinetics of ceforanide (BL S786R) and cefazolin in laboratory animals and humans. *Antimicrob Agent Chemother* **17**: 188–192, 1980.
16. Routledge PA, Barschowsky A, Bjornsson TD, Kitchell BB and Shand DG, Lidocaine plasma protein binding. *Clin Pharmacol Ther* **27**: 347–351, 1980.
17. Fremstad D, Bergerud K, Haffner JFW and Lunde PKM, Increased plasma binding of quinidine after surgery: A preliminary report. *Eur J Clin Pharmacol* **10**: 441–444, 1976.
18. Meffin PJ, Robert EW, Winkle RA, Harapat S, Peters F and Harrison DC, Role of concentration-dependent plasma protein binding in disopyramide disposition. *J Pharmacokin Biopharm* **7**: 29–46, 1979.
19. Zini R, Barre J, Defer G, Jeanniot JP, Houin G and Tillement JP, Protein binding of propisomide. *J Pharm Sci* **74**: 530–533, 1985.
20. Barre J, Zini R and Tillement JP, The binding of erythromycin to proteins in the serum of humans during various infections. *Infection* **10**: Suppl. 2, 113–115, 1982.
21. Sjoholm I, Ekman B, Kober A, Ljungstedt-Pahlman I, Seiving B and Sjodin T, Binding of drugs to human serum albumin. XI The specificity of three binding sites as studied with albumin immobilized in microparticles. *Mol Pharmacol* **16**: 767–777, 1979.
22. Fehske KJ, Muller WE and Wollert U, The location of drug binding sites in human serum albumin. *Biochem Pharmacol* **30**: 687–692, 1981.
23. Maruyama K, Awazu S, Nishigori H and Iwatsuru M, Effects of fatty acids on the specific drug-binding sites of human serum albumin. *Chem Pharm Bull* **34**: 3394–3402, 1986.
24. Tillement JP, Houin G, Zini R, Urien S, Albengres E, Barre J, Lecomte M, D'Athis Ph and Sebille B, The binding of drugs to blood plasma macromolecules: recent advances and therapeutic significance. *Adv. Drug Res* **13**: 59–94, 1984.
25. Berde CB, Hudson BS, Simoni RD and Sklar LA, Human serum albumin. Spectroscopic studies of binding and proximity relationships for fatty acids and bilirubin. *J Biol Chem* **254**: 391–400, 1979.
26. Goodman DS, The interaction of human serum albumin with long-chain fatty acid anions. *J Am Chem Soc* **80**: 3892–3898, 1958.
27. Brodersen R, Honore B and Larsen FG, Serum albumin. A non saturable carrier. *Acta Pharmacol Toxicol* **54**: 129–133, 1984.

28. Jacobsen J, Binding of bilirubin to human serum albumin. Determination of the dissociation constants. *FEBS Lett* **5**: 112–114, 1969.
29. Jacobsen C, Chemical modification of the high affinity bilirubin binding site of human serum albumin. *Eur J Biochem* **27**: 513–519, 1972.
30. Wilding G, Feldhoff RC and Vesell ES, Concentration-dependent effects of fatty acids on warfarin binding to albumin. *Biochem Pharmacol* **26**: 1143–1146, 1977.
31. Brunner F, Zini R and Tillement JP, Plasma protein binding of metbufen, a new non steroidal anti-inflammatory drug in humans. *Int J Clin Pharmacol Ther Toxicol* **22**: 134–139, 1984.
32. Iwatsuru M, Nishigori H and Maruyama K, The classification of drugs on the basis of the drug-binding sites on human serum albumin. *Chem Pharm Bull* **30**: 4489–4493, 1982.
33. Maruyama K, Harada S, Nishigori H and Iwatsuru M, Classification of drugs on the basis of bilirubin-displacing effect on HSA. *Chem Pharm Bull* **32**: 2414–2420, 1984.
34. Takeda N, Niwa T, Tatematsu A and Suzuki M, Identification and quantification of a protein-bound ligand in uremic serum. *Clin Chem* **33**: 682–685, 1987.
35. Chakrabarti SK, Cooperativity of warfarin binding with human serum albumin induced by free fatty acid anion. *Biochem Pharmacol* **27**: 739–743, 1978.