STRUCTURE-BINDING RELATIONSHIP AND BINDING SITES OF CEPHALOSPORINS IN HUMAN SERUM ALBUMIN

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(Received for publication January 29, 1992)

The binding of some cephalosporins to human serum albumin (HSA) was studied by an ultrafiltration technique. Changes in C-3 side chain resulted in marked changes in the binding to HSA, but changes in C-7 side chain did not. Cephalosporins were classified into three groups by C-3 side chain: (i) Cationic side chain with low affinity for HSA; (ii) anionic side chain with high affinity for HSA; (iii) non ionized side chain, in which binding to HSA was dependent on lipophilicity. These findings suggest that electrostatic and hydrophobic forces play a role in the binding affinity of cephalosporins for HSA. The binding of cephalosporins with high HSA affinity was displaced significantly by warfarin but not by phenylbutazone, L-tryptophan, or diazepam. The interaction of the cephalosporins with high affinity for HSA with chemically modified HSA was investigated to clarify the amino acid residues of HSA involved in the cephalosporin binding sites. The binding of the cephalosporins decreased remarkably with the modification of the tyrosine residues. These results suggest that the binding site of cephalosporins is located in the vicinity of warfarin binding site rather than benzodiazepine binding site and that tyrosine residues are involved in the cephalosporin binding site.

There are numerous reports on the binding of cephalosporins to serum protein and the effect of serum protein binding on their pharmacological properties. Cephalosporins are known to bind reversibly to human serum albumin (HSA) to different extents depending on changes in the side chain structure. This binding can affect bacteriological activity[1,2] and drug distribution in the body[3~5]. The therapeutic efficacy of cephalosporins is, therefore, affected by their protein binding[6,7]. On the other hand, there have been a few experiments on the chemical nature of the interactions and the binding sites of cephalosporins[8~10], and the structure-binding relationship has not been clarified.

In our experiments, we measured the binding rates of twenty cephalosporin derivatives to HSA by the ultrafiltration method to clarify the relationship between the binding to HSA and the structure of side chain. Moreover, to elucidate the nature of the binding sites of cephalosporins on HSA, we investigated the effects of some specific marker ligands of HSA and the chemical modification of HSA on the binding of cephalosporins.

Materials and Methods

Antimicrobial Agents

The following agents were used: Cefodizime (CDZM, Taiho Pharmaceutical Co., Ltd.), ceftriaxone (CTRX, Hoffman-La Roche Inc.), ceftizoxime (CZX, Fujisawa Pharmaceutical Co., Ltd.), cefotaxime (CTX, Chugai Pharmaceutical Co., Ltd.), cefmenoxime (CMX, Takeda Chemical Industries, Ltd.), cefuzonam (CZON, Lederle Japan, Ltd.), cephalothin (CET, Shionogi & Co., Ltd.), cefamandole (CMD,

Shionogi), cefoperazone (CPZ, Toyama Chemical Co., Ltd.), cefpiramide (CPM, Sumitomo Pharmaceutical Co., Ltd.), cephaloridine (CER, Shionogi), ceftazidime (CAZ, Glaxo Japan, Ltd.), cefpirome (CPR), cefpodoxime (CPDX), FR 17522, FR 81335, FR 14003, FR 16991, FR 14329, and FR 14431. The last eight cephalosporins were synthesized in our laboratories.

Albumin

Essentially fatty acid-free human serum albumin was obtained from Sigma (St. Louis, U.S.A.).

Marker Ligands

Warfarin was obtained from Sigma. Phenylbutazone and L-tryptophan were obtained from Nacalai Tesque (Kyoto, Japan). Diazepam was synthesized in our laboratories.

Agents for Chemical Modification of HSA

O-Nitrophenylsulfenyl chloride (NSP), tetranitromethane (TNM), and acetylsalicylic acid (ASA) were obtained from Nacalai Tesque.

Experimental Conditions of the Binding to HSA

All experiments of the binding to HSA were carried out at 37°C for 1 hour. All solutions were prepared with sodium phosphate buffer (1/15 M, pH 7.4, with 50 mM NaCl).

Measurement of Antibiotic Concentration

Antibiotic concentrations were measured by the disk plate diffusion method or HPLC (LC-4A model, Shimadzu, Kyoto, Japan). The disk plate diffusion assay was performed by using *Bacillus subtilis* ATCC

Table 1. Microbiological assay method used in determination of the binding rate of the cephalosporins to HSA[a].

Test organism	Test medium	Compound	Detection limit (μg/ml)	Sensitivity[b] (mm·(μg/ml)$^{-1}$)
Bacillus subtilis ATCC 6633	Sodium citrate medium	FR 17522	0.78	9.12
		CPR	0.39	7.68
		CZX	1.56	7.68
		CTX	0.78	8.00
		CET	0.1	9.57
		CMD	0.2	9.93
		CER	0.1	7.93
Escherichia coli ATCC 39188	Nutrient agar	FR 81335	0.78	6.46
		CDZM	0.2	6.99
		CTRX	0.025	5.51
		FR 14003	0.1	6.40
		CPDX	0.05	6.57
		FR 16991	0.1	5.65
		CMX	0.013	5.94
		CZON	0.025	4.63
		FR 14329	0.05	5.94
		FR 14431	0.05	5.53
		CPZ	0.025	4.83
		CPM	0.1	6.06
		CAZ	0.1	5.95

[a] The binding to HSA presented in Tables 2 and 3 was evaluated by microbiological assay as described in the text.

[b] In general, the relationship between concentration of antibiotic (x) and diameter of growth inhibition zone (y) in disk plate diffusion assay is expressed by the following equation: $y = a \cdot \log x + b$, where a and b are the slope and the intercept, respectively. Sensitivity was expressed by the slope of standard curve.

6633 as the test organism and sodium citrate agar (sodium citrate 0.8%, Polypepton 0.5%, beef extract 0.3%, agar 1.0%) as the test medium, or *Escherichia coli* ATCC 39188 as the test organism and nutrient agar (Difco Laboratories, Detroit, U.S.A.) as the test medium. The assay condition, the limit of detection, and the sensitivity are summarized in Table 1. The serial two-fold diluents for the standard curves were prepared with the phosphate buffer described above within the range of 50 μg/ml to the limit of detection. The experimental conditions for measurement by HPLC were as follows: Samples were run on a column (150 mm × 4.6 mm) of TSK gel ODS-80T$_M$ (TOSOH, Tokyo, Japan) at an ambient temperature and a flow rate of 0.8 ml/minute. Composition of the mobile phase was changed according to the purpose. The eluate was monitored at 254 nm. The diluents for the standard curves were prepared in the same way as the microbiological assay within the range of 0.2 to 50 μM. The limits of detection of the cephalosporins (CZON, CMX, CTRX, and FR 14003) in HPLC assay were 0.2 to 0.39 μM.

Binding of Cephalosporins to HSA

The binding of cephalosporins to HSA was determined by the ultrafiltration method, using a HSA concentration of 4% according to the results of INOKAWA *et al.*[11] and 10 or 50 μg/ml of the drugs. The HSA-drug mixture was introduced into a Visking cellulose tubing (size 8/32, Sanko Pure Chemical, Osaka, Japan). The tubing was hung in a 10-ml plastic tube and centrifuged at 1,000 × g for 40 minutes. The reference experiment was performed by using the phosphate buffer in place of HSA. The concentration of drug in the ultrafiltrate was measured by the disk diffusion assay. The ultrafiltrates of cephalosporins with low level of HSA binding at a concentration of 50 μg/ml were diluted with the phosphate buffer by two- or four-fold. The binding rate (percent bound [P]) was calculated by the following equation: $P = 100(1 - X/Y)$, where X and Y are the concentrations of drug in the ultrafiltrate of the sample and the reference, respectively. The cephalosporins tested in this study were stable in 4% HSA solution and the recoveries were above 90% after incubation for 1 hour at 37°C.

Hydrophobic Character of Cephalosporins

The hydrophobic character of cephalosporins was evaluated by the retention time of reverse phase HPLC described above on the basis of the results of YOSHIMURA *et al.*[12], injecting 10 μl of antibiotic solution. The mobile phase consisted of 30 mM sodium phosphate buffer, pH 7.4 and CH_3CN (88 : 12, v/v).

Displacement of Cephalosporins from HSA by Specific Marker Ligands

Warfarin and phenylbutazone, and L-tryptophan and diazepam were used as the specific marker ligands of site I and site II, respectively[13~16]. Displacement of cephalosporins from HSA by these specific marker ligands was investigated by the ultrafiltration method, using a HSA concentration of 50 and 500 μM of cephalosporins to ensure that the drugs were bound mostly to their high affinity site. The marker ligands were added to a 1 : 1 molar ratio with HSA so that binding to nonspecific sites was kept to a minimum. In the displacement experiment by diazepam, the concentrations of HSA, cephalosporins, and diazepam were reduced to 100, 10, and 100 μM, respectively because of low solubility of diazepam in water. 0.3 ml of the HSA-drug-marker ligand mixture was introduced into an ultrafiltration unit "Ultra Free" (Japan Millipore Limited, Tokyo, Japan) and centrifuged at 1,300 × g for 5 minutes. The concentrations of the drugs in the ultrafiltrates were determined by HPLC. The mobile phase consisted of 30 mM sodium phosphate buffer, pH 7.4 and CH_3CN. The composition ratio of them was changed in order that retention time of each of the compounds was about 10 minutes. The extent of binding of cephalosporins to HSA was evaluated by the value of r/A, where r and A are the moles of drug bound per mole of HSA and the molar concentration of the free drug, respectively.

Binding of CZON and CTRX to Chemically Modified HSA Derivatives

The tryptophan residue was modified by NSP according to JACOBSEN[17] (NSP-HSA). 22-fold molar excess of reagent dissolved in acetic acid was added to HSA in 20% aqueous acetic acid under stirring. The reaction time was 5 hours at 0°C. The tyrosine residue was modified by TNM according to the method of SOKOLOVSKY *et al.*[18] and FEHSKE *et al.*[19] (TNM-HSA). 15 molar excess of TNM dissolved in ethanol was added to HSA in 50 mM Tris buffer adjusted to pH 8.0 under stirring. The reaction was carried out at room temperature for 2 hours. The lysine residue was modified by ASA according to HAWKINS *et al.*[20]

(ASA-HSA). HSA (0.1 mM) was incubated at 37°C for 24 hours with ASA (0.5 mM) in phosphate buffer, pH 7.4. After modification, NSP-HSA and TNM-HSA were purified by dialysis against distilled water for 60 hours at 4°C, and then lyophilized according to FEHSKE *et al.*[19]. ASA-HSA was dialyzed at 4°C for 48 hours against multiple changes of 0.15 M NaCl containing 50 mM phosphate buffer, pH 7.4 and finally against distilled water, and then lyophilized according to CHIGNELL *et al.*[21]. In control experiments HSA was treated in the same way but without adding the reagents for chemical modification and used as control HSA. The degree of tryptophan[22] and tyrosine[18] residues modification of the HSA derivatives was determined spectrophotometrically. The numbers of tryptophan and tyrosine residues modified were 1.2 and 4.3, respectively, and these results were consistent with those of other investigators[17,19]. The degree of lysine residue modification was examined by the binding of phenylbutazone to ASA-HSA. The modification by ASA increased the affinity of the protein for phenylbutazone, and this result was in agreement with that of CHINGELL *et al.*[21].

The binding of CZON and CTRX to chemically modified HSA derivatives was determined by the ultrafiltration method with Ultra Free described above using a HSA concentration of 500 μM and varying concentrations of the drugs. The concentration of drug in the ultrafiltrate was measured by HPLC. The mobile phase consisted of 30 mM citrate and CH_3CN. The composition ratio of them was changed in order that retention time of each of the compounds was about 10 minutes.

Results were plotted by the method of KLOTZ and HUNSTON[23]. The binding parameters were calculated according to the following formula, based on the Scatchard model consisting of two kinds of binding site[23]:

$$\frac{r}{A}=\frac{n_1k_1}{1+k_1A}+\frac{n_2k_2}{1+k_2A} \qquad (1)$$

where r is the number of moles of drug bound per mole of HSA; n_1 and n_2, the number of binding sites in each class; k_1 and k_2, the association constants for these sites; and A, the concentration of free drug.

Displacement of CZON from HSA by CTRX

The binding of CZON to HSA was determined in the presence of the fixed free concentration of CTRX (final free concentration of CTRX; 9~11 μM) by the ultrafiltration method described above, using a HSA concentration of 500 μM and varying concentration of CZON. The concentrations of CZON and CTRX in the ultrafiltrates were determined by HPLC. Results were plotted by the method of KLOTZ and HUNSTON[23]. The binding parameters of CZON to HSA were calculated according to the formula (1).

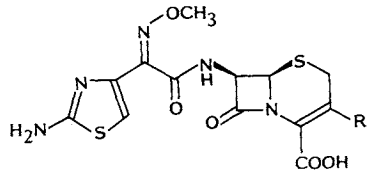
Results

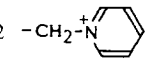
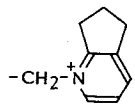
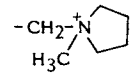
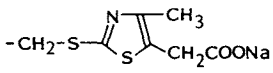
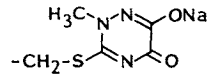
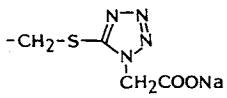
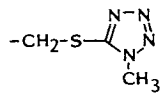
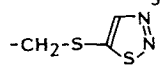
Structure-binding Relationship of Cephalosporin Antibiotics to HSA

The binding of twenty cephalosporin antibiotics to 4% HSA at concentrations of 10 and 50 μg/ml was determined by the ultrafiltration method to clarify the structure-binding relationship of cephalosporins to HSA. The effect of C-3 side chain on the binding was investigated using cephalosporins having the same C-7 side chain, aminothiazolyl-methoxyimino-acetamide group. As shown in Table 2, the extent of the binding of the drugs to HSA varied greatly from 0 to 98.8% with changes in C-3 side chain. The drugs with cationic C-3 side chain showed low extents of HSA binding. On the contrary, the drugs with anionic C-3 side chain showed high extents of HSA binding. The extent of the binding of the drugs with non ionized C-3 side chain was dependent on their lipophilicity, which was evaluated by the retention time of reverse phase HPLC.

The effect of C-7 side chain on the binding was investigated using four groups of cephalosporins. The results are shown in Table 3. As a whole, the effect of C-7 side chain on the binding was less remarkable than that of C-3 side chain. Change in the extent of the binding to HSA was small in the cephalosporins having a highly lipophilic C-3 side chain such as (1-methyl-1(*H*)-tetrazole-5-yl)thiomethyl group or a

Table 2. Effect of C-3 side chain on the binding of cephalosporins having an aminothiazolyl-methoxyimino-acetamide group at C-7 side chain to human serum albumin.



Compound	R	Rt (minutes)	Binding rate (%) at a concentration of	
			50 μg/ml	10 μg/ml
FR 17522		4.8	0.9	0.7
CPR		8.0	0	1.4
FR 81335		5.0	0	5.6
CDZM		5.7	76.8	83.6
CTRX		3.6	95.1	96.2
FR 14003		3.8	97.5	98.8
CZX	$-H$	4.6	6.0	22.9
CPDX	$-CH_2OCH_3$	5.6	33.3	34.6
CTX	$-CH_2OCOCH_3$	8.2	47.1	35.4
FR 16991	$-CH_2=CH_2$	7.6	49.0	56.5
CMX		11.1	79.9	82.6
CZON		25.9	88.5	92.7

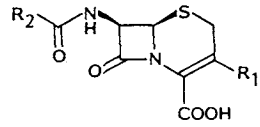
The binding to HSA was determined by the ultrafiltration method with a Visking tubing and the concentrations of the cephalosporins in ultrafiltrates were measured by the microbiological assay as described in the text. Hydrophobic character of the cephalosporins was evaluated by the retention time of HPLC.

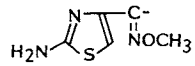
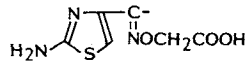
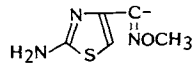
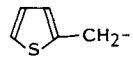
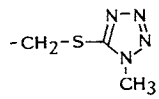
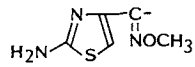
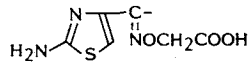
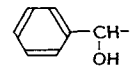
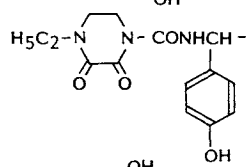
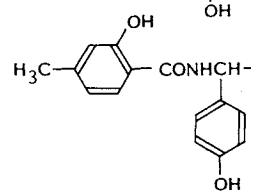
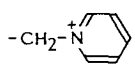
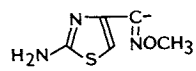
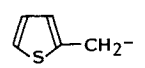
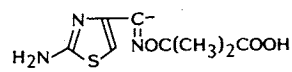
cationic C-3 side chain such as pyridinomethyl group. In the cephalosporins having a low lipophilic C-3 side chain such as hydrogen or a moderately lipophilic C-3 side chain such as acetoxymethyl group, the extent of the binding to HSA varied by about two-fold with changes in C-7 side chain.

Displacement of Cephalosporins from HSA by Specific Marker Ligands

The binding of drugs to HSA is generally assumed to be rather nonspecific than specific, but some specific binding sites have been demonstrated on HSA molecule[13]. To clarify the binding sites of cephalosporins on HSA, displacement experiments by the marker ligands of site I (warfarin and

Table 3. Effect of C-7 side chain on the binding of cephalosporins to human serum albumin.



Compound	R_1	R_2	Rt (minutes)	Binding rate (%) at a concentration of	
				50 μg/ml	10 μg/ml
CZX	-H		4.6	6.0	22.9
FR 14329	-H		2.3	51.9	52.2
CTX	$-CH_2OCOCH_3$		8.2	47.1	35.4
CET	$-CH_2OCOCH_3$		36.9	67.4	74.0
CMX			11.1	79.9	82.6
FR 14431			3.8	85.6	84.9
CMD			25.8	88.5	85.4
CPZ			8.3	89.3	91.1
CPM			16.1	97.6	98.8
FR 17522			4.8	0.9	0.7
CER			12.7	0	14.0
CAZ			3.0	2.7	12.3

The binding to HSA was determined by the ultrafiltration method with a Visking tubing and the concentrations of the cephalosporins in ultrafiltrates were measured by the microbiological assay as described in the text. Hydrophobic character of the cephalosporins was evaluated by the retention time of HPLC.

phenylbutazone) and site II (L-tryptophan and diazepam) were performed. The cephalosporins having a highly lipophilic substituent (CZON and CMX) or an anionic substituent (CTRX and FR 14003) at C-3 position were chosen because these compounds showed high HSA binding. As shown in Table 4, the

Table 4. Effect of warfarin, phenylbutazone, L-tryptophan, and diazepam on binding of cephalosporins to HSA.

Compound	Marker ligand	Binding of cephalosporins to HSA (% control)	Compound	Marker ligand	Binding of cephalosporins to HSA (% control)
CZON	Warfarin	62.8	CTRX	Warfarin	60.3
	Phenylbutazone	90.8		Phenylbutazone	98.0
	L-Tryptophan	100		L-Tryptophan	107
	Diazepam	89.6		Diazepam	110
CMX	Warfarin	51.0	FR 14003	Warfarin	49.3
	Phenylbutazone	94.7		Phenylbutazone	91.4
	L-Tryptophan	106		L-Tryptophan	102
	Diazepam	96.9		Diazepam	110

The binding to HSA was determined by the ultrafiltration method and the concentrations of the cephalosporins in ultrafiltrates were measured by HPLC.

The extent of binding of the cephalosporins to HSA was evaluated by the value of (r/A) as described in the text.

r; moles of drug bound per mole of HSA, A; molar concentration of the free drug.

binding of these cephalosporins to HSA was significantly displaced by warfarin. The other marker ligands did not affect the binding of the cephalosporins to HSA in contrast with warfarin. Although two kinds of cephalosporins which were different in their chemical properties of C-3 side chain were chosen as the test antibiotics, a similar pattern of displacement by the four marker ligands was seen. These results indicate that the binding site of the cephalosporins tested on HSA is located in the vicinity of warfarin binding site and suggest that these cephalosporins may be bound to the same site of HSA despite the difference of chemical nature of C-3 side chain.

The Binding of CZON and CTRX to Chemically Modified HSA Derivatives

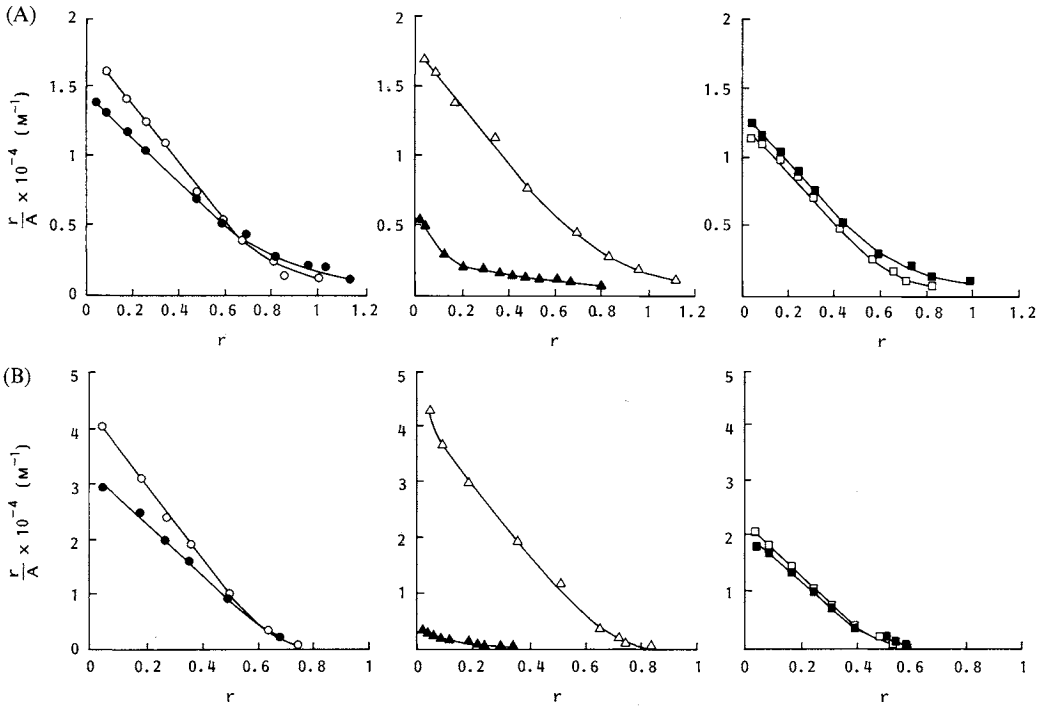
It is accepted that tryptophan, tyrosine, and lysine residues are involved in site I of HSA as demonstrated by the effect of chemical modification of these amino acid residues on the binding of warfarin[24] and phenylbutazone[21]. Therefore, the influence of the modification of these amino acid residues on the binding of cephalosporins was examined for further characterization of the binding site of cephalosporins. CZON and CTRX were chosen as the representative compounds with a highly lipophilic C-3 side chain and an anionic C-3 side chain, respectively.

As shown in Fig. 1, the binding of CZON was markedly and slightly decreased by the modification of tyrosine and tryptophan residue, respectively. Similar results were obtained for CTRX. Curved Scatchard plots were obtained for most of investigated HSA derivatives. They could be resolved into two linear components according to the method of KLOTZ *et al.*[23], each representing one set of binding sites. Therefore, the binding parameters of CZON and CTRX to HSA derivatives were calculated according to the Scatchard model consisting of two kinds of binding sites[23].

The binding parameters of CZON and CTRX to chemically modified HSA derivatives are shown in Table 5. The modification of the lone tryptophan residue with NSP slightly reduced the association constant of the high-affinity binding site of CZON by 37 and 22% in comparison with native HSA and control HSA, respectively, but did not affect the number of high-affinity binding site. The modification of the tyrosine residues with TNM remarkably reduced the number of the high-affinity binding site of CZON by 83 and 86% in comparison with native HSA and control HSA, respectively, but did not so remarkably affect the association constant of the high-affinity binding site. The modification of the lysine residue with

Fig. 1. Scatchard plot of the binding of CZON (A) and CTRX (B) to NSP-HSA, TNM-HSA, and ASA-HSA.

A; Molar concentration of the free drug, *r*; moles of drug bound per mole of albumine, ●; NSP-HSA, ○; NSP-cont-HSA, ▲; TNM-HSA, △; TNM-cont-HSA, ■; ASA-HSA, □; ASA-cont-HSA.

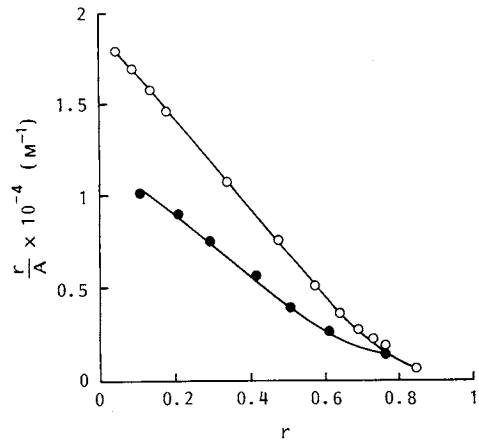


The binding to HSA derivatives was determined by the ultrafiltration method and the concentrations of the cephalosporins in ultrafiltrates were measured by HPLC.

ASA affected neither the association constant nor the number of the high-affinity binding site of CZON. Similar results were obtained for CTRX. That is, a slight reduction in the association constant of high-affinity binding site by the tryptophan residue modification, a remarkable reduction in the number of high-affinity binding site by the tyrosine residues modification, and no effects of the lysine residue modification on the binding parameters were observed. In addition to these changes in the parameters, a remarkable reduction in the association constant of high-affinity binding site was also observed by the modification of tyrosine residues. As a whole, the variabilities of the binding parameters of low-affinity binding site were large, especially in the cases of the binding of CZON to NSP-cont-HSA and the binding of CTRX to TNM-HSA and TNM-cont-HSA. Since the affinity to HSA deriva-

Fig. 2. Scatchard plot of the binding of CZON alone and in the presence of CTRX to HSA.

A; Molar concentration of the free drug, *r*; moles of drug bound per mole of albumine, ○; CZON, ●; CZON+CTRX.



The binding to HSA derivatives was determined by the ultrafiltration method and the concentrations of the cephalosporins in ultrafiltrates were measured by HPLC.

Table 5. Binding parameters of CZON and CTRX to NSP-HSA, TNM-HSA, and ASA-HSA.

Compound	Albumin	n_1	$10^{-4} \times k_1$ (M^{-1})	n_2	$10^{-4} \times k_2$ (M^{-1})
CZON	Native HSA	0.65±0.08	2.86±0.32	0.25±0.07	0.42 ±0.17
	NSP-HSA	0.74±0.21	1.81±0.50	0.84±0.27	0.12 ±0.15
	NSP-cont HSA	0.77±0.09	2.33±0.35	2.94±26.3	0.01 ±0.11
	TNM-HSA	0.11±0.06	3.00±2.00	1.26±0.14	0.12 ±0.04
	TNM-cont HSA	0.80±0.05	2.17±0.20	1.21±0.53	0.05 ±0.04
	ASA-HSA	0.66±0.09	1.96±0.38	1.76±2.70	0.03 ±0.05
	ASA-cont HSA	0.69±0.06	1.84±0.24	1.02±3.80	0.02 ±0.08
CTRX	Native HSA	0.54±0.08	8.62±2.73	0.64±0.22	0.12 ±0.11
	NSP-HSA	0.66±0.10	5.09±0.81	0.23±1.74	0.11 ±1.40
	NSP-cont HSA	0.58±0.03	7.31±0.47	0.21±0.03	0.54 ±0.20
	TNM-HSA	0.23±0.10	1.62±0.70	7.96±483	0.003±0.19
	TNM-cont HSA	0.70±0.03	5.87±0.54	3.25±39.1	0.004±0.06
	ASA-HSA	0.41±0.11	4.98±1.42	0.23±0.09	0.52 ±0.46
	ASA-cont HSA	0.37±0.10	6.09±1.58	0.22±0.09	0.85 ±0.55

Each value represents the estimated parameter ±SD based on the nonlinear least-squares fitting of data in Fig. 1. The association constants k_1 and k_2 and the number of binding sites on the albumin molecule n_1 and n_2 were calculated using the Scatchard model consisting of two kinds of binding sites.

Table 6. Binding parameters of CZON alone and in the presence of CTRX to HSA.

Compound	n_1	$10^{-4} \times k_1$ (M^{-1})	n_2	$10^{-4} \times k_2$ (M^{-1})
CZON	0.65±0.08	2.86±0.32	0.25±0.07	0.42±0.17
CZON+CTRX	0.68±0.16	1.80±0.45	1.24±12.4	0.02±0.29

Each value represents the estimated parameter ±SD based on the nonlinear least-squares fitting of data in Fig. 2. The association constants k_1 and k_2 and the number of binding sites on the albumin molecule n_1 and n_2 were calculated using the Scatchard model consisting of two kinds of binding sites.

tives was very low in these cases, the binding parameters could not be correctly estimated.

These results indicate that the tyrosine residues play an important role in the interaction between these cephalosporins and HSA but the tryptophan and the lysine residues do not.

Displacement of CZON from HSA by CTRX

The results shown in Tables 4 and 5 suggest that CZON and CTRX are bound to the same site on HSA. To confirm this possibility, the binding of CZON to HSA was investigated in the presence of the fixed free concentration of CTRX as described in the Materials and Methods section. As shown in Fig. 2, a competitive type displacing effect of CTRX on the binding of CZON to HSA was observed. The binding parameters of CZON calculated from the Scatchard plot in Fig. 2 are shown in Table 6. The association constant of high-affinity binding site of CZON was reduced by the addition of CTRX, but the number of high-affinity binding site of CZON was not affected.

This competitive inhibition of the binding of CZON by CTRX suggests that these compounds are bound to the same site on HSA.

Discussion

There are some reports on the chemical nature of the binding and the structure-binding relationship

of β-lactam antibiotics to serum albumin, but the results are contradictory. BIRD *et al.*[25] demonstrated that the extent of binding of penicillins to HSA increased with the hydrophobic character of the side chain. However, the studies of KEEN[26] and PHILLIPS *et al.*[27] indicate that ion binding can be achieved between the carboxyl group of penicillins and the cationic sites of bovine serum albumin and that this ion binding may facilitate other short-range hydrophobic interactions. BRIAND *et al.*[9] also showed that binding forces involved in the interaction between HSA and some cephalosporins were principally electrostatic and the major binding site for cephalosporins with high HSA affinity was the electron-rich heterocycle fixed on the methylene at position 3. These results are supported by the findings of NOUDA *et al.*[10], who demonstrated that the correlation between lipophilicity and binding affinity of cephalosporins to HSA was very poor. For these reasons, we studied the structure-binding relationship and the binding mechanism of cephalosporins to HSA.

The extent of the binding of cephalosporins to HSA was greatly affected by the chemical property of C-3 side chain. From the results shown in Table 2, it is possible to classify the cephalosporins into three groups by their C-3 side chain as follows: (i) Molecules with cationic C-3 side chain, which had low levels of HSA binding; (ii) molecules with anionic C-3 side chain, which had high levels of HSA binding; and (iii) molecules of non-ionized C-3 side chain, in which a correlation was seen between lipophilicity and the extent of HSA binding. On the other hand, the effect of C-7 side chain on the binding to HSA was less remarkable than that of C-3 side chain although C-7 side chain affected the binding of the drugs having a low or a moderately lipophilic C-3 side chain to some extent. These findings suggest that the binding of cephalosporins to HSA is determined mostly by the chemical property of C-3 side chain. Our results were consistent with the findings of BRIAND *et al.*[9] in that C-3 side chain plays an important role in the binding to HSA, and with those of NOUDA *et al.*[10] in that lipophilicity is not a sole factor determining the binding to HSA because the molecules with anionic C-3 side chain showed high levels of HSA binding despite of their low lipophilicity at pH 7.4 in our study, and finally with those of BIRD *et al.*[25] in that the binding of the molecules with non-ionized C-3 side chain was dependent on lipophilicity of C-3 side chain.

The results on the structure-binding relationship suggest that the cephalosporins interact with HSA by ion binding or hydrophobic binding. Therefore, we investigated the binding sites of the cephalosporins having a highly lipophilic C-3 side chain or an anionic C-3 side chain to clarify the difference of the binding mechanism of these cephalosporins. The cephalosporins tested were significantly displaced from HSA by warfarin but not by the other marker ligands. Moreover, there was not a significant difference in the displacement pattern of the cephalosporins by four marker ligands despite the difference of chemical property of their C-3 side chain. These findings indicate that the cephalosporins are bound to site I rather than site II. It is accepted that site I actually is a larger binding area, consisting of the overlapping binding sites of warfarin and azapropazone because the modification of the lone tryptophan residue reduces the binding of warfarin but does not the binding of phenylbutazone and of azapropazone[13]. Our results indicate that the cephalosporins are bound to the warfarin binding site in site I. NOUDA *et al.*[10] demonstrated that CET and cefazolin (CEZ) were displaced from HSA by warfarin and phenylbutazone, and suggest that CET and CEZ are bound to site I. Our results are consistent with their findings in that cephalosporins were bound to site I, but they were contradictory in that phenylbutazone did not affect the binding in our study.

It seems that the binding site of lipophilic cephalosporins differ from that of anionic cephalosporins because the binding forces involved in the interaction with HSA are thought to be different from each other. However, there was not a significant difference between the two kinds of cephalosporins in displacement by specific marker ligands. Therefore, we investigated amino acid residues involved in the binding of these cephalosporins for further characterization of the binding sites. The extents of the binding of CZON (lipophilic) and CTRX (anionic) were remarkably reduced by the modification of the tyrosine residues, but the effect of the modification of the tryptophan or the lysine residue on their binding was not so remarkable. Our results are consistent with the findings of NOUDA *et al.*[10] who demonstrated that the extents of the binding of CET and CEZ were greatly reduced by the modification of the tyrosine residue but not by the modification of the tryptophan residue. These findings indicate that the tyrosine residues are involved in the high-affinity binding site of CZON and CTRX. The binding parameters of low-affinity site could not be correctly estimated as shown by large variabilities. However, it is thought that the binding to this site is not so important to clarify the binding characteristic because the association

constants are very low in comparison with those of high-affinity site and it might be nonspecific rather than specific.

The results on the binding to the chemically modified HSA derivatives suggest that CZON and CTRX may be bound to the same site on HSA. This possibility was supported by the result of mutual displacing experiment, in which a competitive type displacing effect was observed between CZON and CTRX. However, this result is intuitively unsatisfying in some ways because it is supposed that CTRX interacts with basic amino acid residues rather than aromatic amino acids such as tyrosine and tryptophan. MELOUN *et al.*[28] determined the complete amino acid sequence of HSA and demonstrated that fifty-nine lysines, twenty-four arginines, and sixteen histidines are involved in HSA. WALKER[29] showed that the lysine 199 was modified by the method used in this study and this lysine residue seems to be involved in phenylbutazone binding site[21]. This lysine 199 was not involved in the CTRX binding site but the other lysine residues which were not modified by the method in this study might participate in the binding of CTRX. ROOSDROP *et al.*[30] and GAMBHIR *et al.*[31] demonstrated that arginine and histidine residues were involved in site II, although it has not been reported yet that these amino acids are involved in site I. However, we can not exclude the possibility that these amino acids are located in the vicinity of CTRX binding site. Although our results suggest that CZON and CTRX are bound to the same site or the very close site to each other, further characterization of their binding sites is necessary to clarify whether they are bound to the exactly same site.

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