Interactions of Aldosterone Antagonist Diuretics with Human Serum Proteins

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Received October 11, 1996; accepted January 14, 1997

Purpose. The purpose of this study was to investigate the binding mechanism of aldosterone antagonist diuretics with human serum proteins, human serum albumin (HSA) and α_1-acid glycoprotein (AGP), as well as to identify the binding sites of the drugs on these proteins.
Methods. Binding activities of spironolactone (SP) and its pharmacologically active metabolite canrenone (CR) to serum and serum protein were examined by ultrafiltration and spectroscopic techniques. The data for the binding of these drugs to HSA were analyzed on the basis of a theoretical model of simultaneous binding of the ligands.
Results. The binding percentages of antagonist diuretics SP and CR to human serum proteins were 88.0% and 99.2%, respectively, at therapeutic concentrations. SP bound strongly and almost equally to both HSA and AGP, but CR bound strongly only to HSA. In addition, the displacement results found using fluorescent probes and ultrafiltration methods demonstrated that SP bound to site I, particularly to the warfarin region on HSA, and to the basic binding site on AGP, while CR bound to the warfarin region on HSA.
Conclusions. The limited results presented here stress the need for caution on coadministration of acidic drugs which bind to the warfarin region on HSA and basic drugs which bind to AGP with SP and its metabolite CR.

KEY WORDS: spironolactone; canrenone; drug interaction; human serum albumin; α_1-acid glycoprotein.

INTRODUCTION

The aldosterone antagonist spironolactone [7-(Acetylthio)-17-hydroxy-3-oxo-pregn-4-ene-21-carboxylic acid γ-lactone, (SP)] is extensively metabolized in humans (1). SP and its metabolites, 7α-thiomethyl spironolactone and canrenone (CR), are known to have antimineralocorticoid activity in man (1). Therefore, it is desirable to determine the serum concentrations of SP and these two metabolites after administration of SP. However, $t_{1/2}$ values of SP and 7α-thiomethyl spironolactone are much shorter than that of CR, suggesting that the relative concentration of CR at steady state will be higher than those of SP or 7α-thiomethyl spironolactone.

Biopharmaceutical studies of SP and its derivatives revealed that they bind extensively to serum protein. This suggests that SP and its metabolites may interact in vivo. However, detailed analyses of SP-serum protein interactions including binding sites have not been reported. A better understanding of SP-serum protein interactions might help to predict drug interactions because SP and CR are frequently coadministered with different drugs such as antibiotics and calcium antagonists.

Thus, the present study was undertaken to investigate the binding mechanism of aldosterone antagonist diuretics with human serum proteins, human serum albumin (HSA) and α_1-acid glycoprotein (AGP), as well as to characterize the binding sites of the drugs on these proteins.

MATERIALS AND METHODS

Materials

HSA (essentially fatty acid-free albumin), IgG (human immunoglobulin) and dansylsarcosin (DNSS) were purchased from Sigma Chemical Co. (St. Louis, NO, U.S.A.). AGP was a gift from Chem-Sera-Therapeutic Research Institutes (Kumamoto, Japan). Spironolactone (SP) and potassium canrenoate (CR) were gifts from Searle Co. (Osaka, Japan). The chemical purities of different aldosterone antagonist diuretics were above 99% as determined by HPLC. Potassium warfarin and verapamil hydrochloride (Eisai Co., Tokyo, Japan), cefazoline sodium (Fujisawa Co., Osaka, Japan), sodium valproate (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan), and phenylbutazone (Ciba Geigy, Summit, NJ, U.S.A.) were obtained as pure substances from the respective manufacturers. All other reagents were of analytical grade. All the buffers used were prepared with 0.067 M phosphate buffer, pH 7.4.

Methods

Fluorescence Method

Fluorescence measurements were obtained using a Jasco FP-770 (Tokyo, Japan). The percentage of displacement of probe was determined according to the method of Sudlow *et al.* (2).

Ultrafiltration Method

Ultrafiltration experiments were performed using Tosoh plastic ultrafiltration apparatus (Kanagawa, Japan). Aliquots of various ratios of drug-HSA (120 μM), AGP (40 μM) or serum protein (4 or 4.9g/100ml equivalent to HSA) mixtures (0.9 or 1.35 ml) were centrifuged at 3300 or 3700 rpm for 15 minutes at 25°C. Adsorption of drugs or probes onto the membrane or apparatus was negligible. No protein leakage was detected during the experiment. The free concentration of drug or displacer was determined by HPLC in a system consisting of a Shimadzu SPD-6A pump, Shimadzu LC-6A UV detector and Shimadzu system controller SCL-6A. A column of LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as a stationary phase for all compounds. To assay the compounds, an UV monitor was used. The UV wavelengths were 250 nm, 245 nm, 285 nm, 285 nm, and 285 nm for SP, DNSS, warfarin, phenylbutazone and CR, respectively. The mobile phases consisted of water-acetonitrile (17:33 v/v) for SP, of water-acetonitrile (227:23 v/v) for warfarin, of water-acetonitrile (9:1 v/v) for DNSS and of water-acetonitrile (221:29 v/v) for CR and phenylbutazone.

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0724-8741/97/0400-0522$12.50/0

UV Spectroscopic Experiments

Difference spectra [UV spectrum (drug-HSA or AGP mixture)-UV spectrum (drug)-UV spectrum (HSA or AGP)] were measured using a pair of 10 mm split compartment-tandem cuvettes.

Data Analysis

Binding parameters were estimated by fitting the experimental data to the following Scatchard equation (3), using a non-linear squares computer program (MULTI program) (4).

$$r = \frac{[D_b]}{[P_t]} = \sum_{i=1}^{j} \frac{n_i K_i [D_f]}{1 + K_i [D_f]} \quad (1)$$

where r is the number of moles of bound drug per protein molecule, $[D_b]$ and $[D_f]$ are the bound and unbound drug concentrations respectively, $[P_t]$ is the total protein concentration, and K_i and n_i are the binding constant and the number of binding sites for the its class of binding sites, respectively. More than 20 points were used for Scatchard analysis. Experimental data were reasonably fitted for Scatchard analysis (AIC (Akaike's information criterion) <60). The simultaneous binding of two ligands was analyzed using a previously reported method (5).

$$r_A = \frac{[A_b]}{[P_t]} = \frac{K_A [A_f] + \chi K_{BA} K_B [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{BA} K_B [A_f][B_f]} \quad (2)$$

$$r_B = \frac{[B_b]}{[P_t]} = \frac{K_B [B_f] + \chi K_{AB} K_A [A_f][B_f]}{1 + K_A[A_f] + K_B[B_f] + \chi K_{AB} K_B [A_f][B_f]} \quad (3)$$

where K_A and K_B are the binding constants of ligand A and B, $[A_f]$ and $[B_f]$ are the free concentrations of ligand A and B, and $[A_b]$ and $[B_b]$ are the bound concentrations of ligand A and B, respectively. χ is a coupling constant, K_{BA} is the binding constant of ligand A in the presence of ligand B, and K_{AB} is the binding constant of ligand B in the presence of ligand A. Using these equations, it is possible to calculate the theoretical values of χ. The interaction mode of the ligands on a macromolecule can be evaluated by the sign and magnitude of the value of χ. For example, if ligand A and B are independently bound to a protein, χ is equal to 1. $\chi > 1$ and $0 < \chi < 1$ indicate cooperative and anti-cooperative interaction between ligands, respectively. Competitive displacement between ligands is indicated by $\chi = 0$.

RESULTS

The chemical structures and different physico-chemical properties of SP and CR are shown in Table 1.

Binding of Aldosterone Antagonist Diuretics to Serum and Serum Protein

The binding percentages of antagonist diuretics SP and CR to human serum proteins at therapeutic concentrations were 88.0% and 99.2%, respectively. Binding of drugs to purified serum proteins was also examined. SP bound strongly to both HSA and AGP almost equally, whereas CR bound only to HSA strongly (Fig. 1).

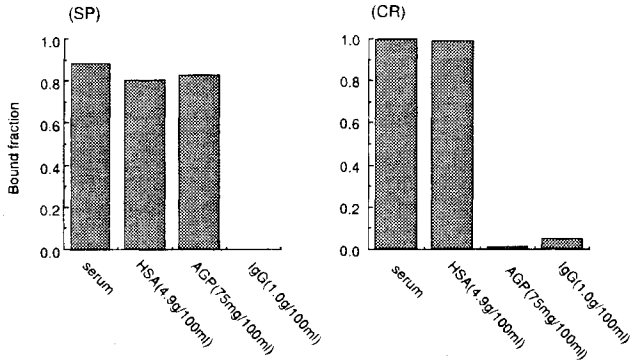


Fig. 1. Binding of SP and CR to serum, HSA, AGP and IgG by ultrafiltration at pH 7.4 and 25°C. The following concentrations were used: serum (as HSA), 4.9g/100ml; HSA, 4.9g/100ml; AGP, 75mg/100ml; IgG, 1g/100ml; SP, 0.5 μM; CR, 30 μM.

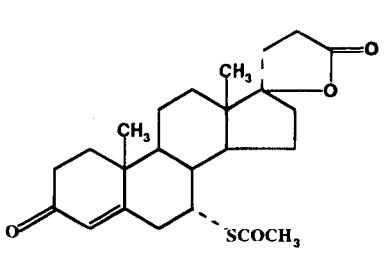
The binding parameters for interactions of drugs with HSA or AGP are shown in Table 2.

Effects of Several Drugs on the Binding of SP or CR to Serum and Serum Protein

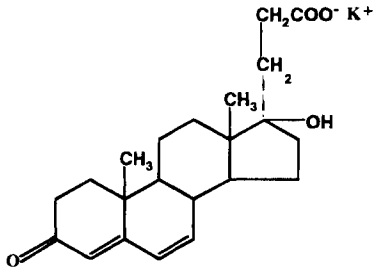
Effects of several acidic and/or basic drugs on the free fractions of SP and CR to serum were examined at respective serum therapeutic concentration levels (Fig. 2). The free fraction of SP was not changed by acidic or basic drugs. The free fraction of CR was increased only by cefazolin among the drugs examined. The interaction of SP and its metabolite CR was not observed in these experimental conditions. The effects of basic drugs on the binding of CR to serum were not examined because CR binds extensively to albumin.

Mutual displacement experiments were carried out and data were analyzed on the basis of a theoretical model of simultaneous binding of two ligands. Figure 3 shows the binding of CR to HSA in the presence of cefazolin (Fig. 3A) and vice versa (Fig. 3B). In both cases, the observed data fitted the theoretical curve well, assuming competitive binding between CR and cefazolin as predicted from the displacement data using the fluorescent probes (data not shown). Cefazolin is considered to bind to site I on HSA (6). Therefore, these results suggest that CR also binds to site I on HSA. To confirm this, further displacement experiments were carried out. As expected, CR was found to bind to site I, especially to the warfarin area on HSA. A competitive interaction was observed for CR-warfarin, anti-cooperative binding was found for CR-phenylbutazone and independent binding was shown for CR- DNSS, a site II probe (Fig. 4). Moreover, to characterize the binding site of CR on HSA, the difference UV spectra of CR bound to HSA were measured. The difference spectrum in the binding of CR to HSA was characterized by one minimum at 303 nm. The absorption of CR produced similar spectra when the CR molecule was dissolved in a solution containing the cationic detergent cetyltrimethylammonium bromide and the non-ionic detergent polyoxyethylene lauryl ether (data not shown), suggesting that the microenvironment for the binding site of CR on HSA is similar to those in the detergents.

Table 1. Chemical Structures and Physicochemical Properties of Aldosterone Antagonists



Spironolactone (SP)



Potassium Canrenoate (CR)

	SP	CR
Partition coefficient[a]	12	3.9
pKa		5.2

[a] Partition coefficient values were calculated based on the partition between phosphate buffer (pH7.4) and 1-octanol saturated with buffer.

Table 2. Binding Parameters of Aldosterone Antagonists to Serum Protein as Determined by Ultrafiltration at pH7.4 and 25°C

	HSA				AGP			
Drug	n_1	$K_1(\times 10^4 M^{-1})$	n_2	$K_2(\times 10^4 M^{-1})$	n_1	$K_1(\times 10^4 M^{-1})$	n_2	$K_2(\times 10^4 M^{-1})$
SP	1.0 ± 0.1	0.3 ± 0.04			1.0 ± 0.1	11.0 ± 0.9		
CR	1.0 ± 0.12	20 ± 1.5	3.0 ± 0.2	0.9 ± 0.12	1.0 ± 0.04	0.5 ± 0.06		

Note: The following concentrations were used; [HSA] = 120 μM, [AGP] = 40 μM, [Drug] = 10 ~ 220 μM. Values represent the means ± SD.

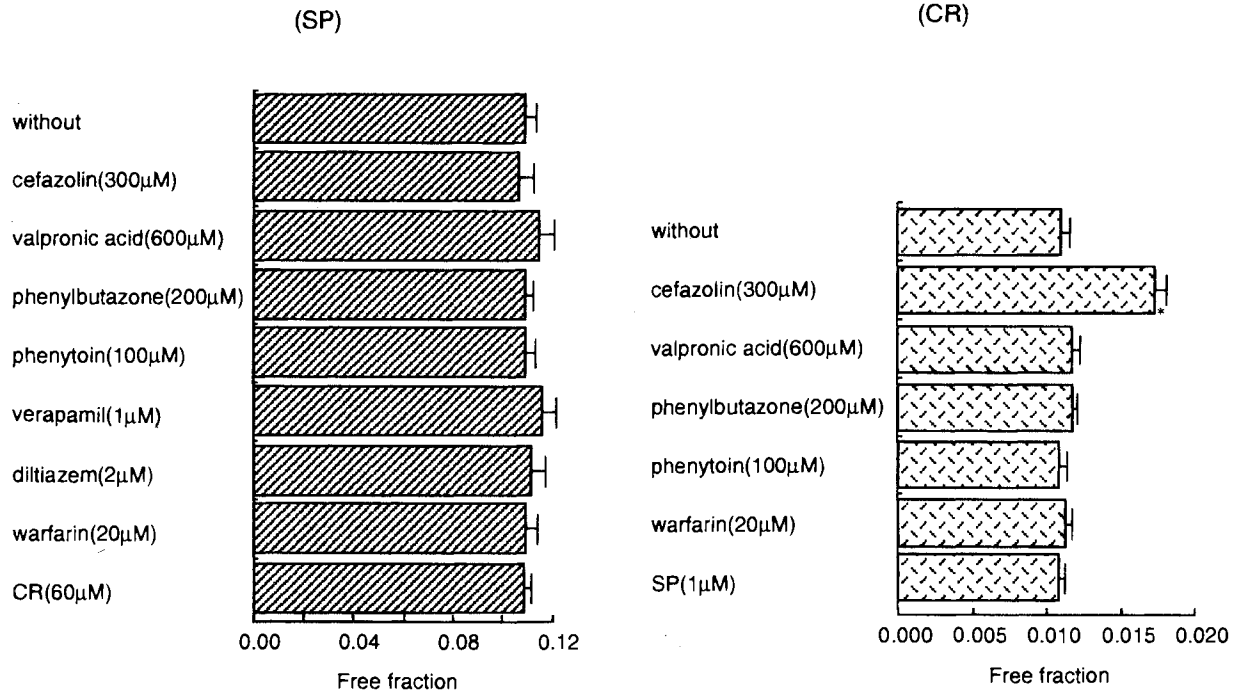


Fig. 2. Different free fractions of SP- and CR-serum systems by the addition of drugs at pH 7.4 and 25°C. The following concentrations were used: serum (as HSA), 4g/100ml; SP, 0.5 μM; CR, 30 μM. Each column is the mean of three experiments ± SD. *$P < 0.001$ vs. control.

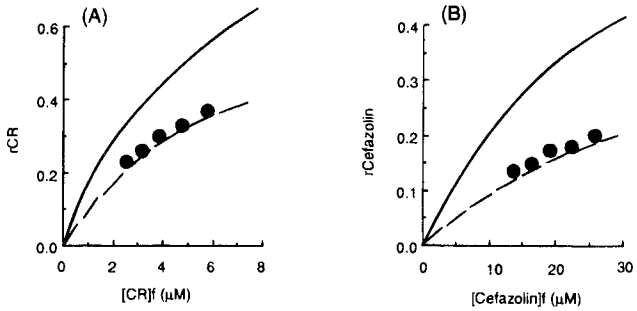


Fig. 3. Binding of CR in the presence of cefazolin (A) to HSA at pH 7.4 and 25°C and vice versa (B). (A) Binding of CR (30–50 μM) to HSA (120 μM) in the presence of cefazolin (100 μM). (B) Binding of cefazolin (30–50 μM) to HSA (120 μM) in the presence of CR (80 μM). ●, Experimental values; _ _ _ _, Theoretical curve assuming competitive binding; _____, Theoretical curve assuming independent binding. All the theoretical curves were constructed by using the n_i and K_i values given in Table 2 (for cefazolin, $n_1 = 1$ and $K_1 = 2.3\times10^4$).

DISCUSSION

Binding percentages of both SP and CR to serum proteins were more than 88% at their therapeutic concentrations. SP bound to both HSA and AGP, while CR bound almost exclusively to albumin. However, the reason is not clear. The different bindings found for these systems can be due to differences in the conformation of the two proteins as well as physical properties including hydrophobicity and molecular structures of the drugs. Particularly, the acetylthioester group in SP and ethyl ester group in CR are thought to affect their binding to HSA and AGP. Anthony *et al.* (7) suggested that SP bound to HSA and γ-globulin. This difference was because they did not examine the binding of SP to AGP. Under physiological conditions, the binding percentage of SP to HSA (80%) is almost the same as that to AGP (82%). When more detailed experiments concerning the SP-HSA and SP-AGP interactions were performed, it became clear that AGP had a much larger affinity for SP than HSA (Fig. 1 and Table 2). Plasma concentrations of AGP and HSA are affected by several diseases; for example, inflammation and renal insufficiency may increase AGP concentrations but decrease HSA levels, leading to the enhancement of drug binding to AGP but a reduction in HSA binding.

The free fraction of CR was significantly increased by the addition of cefazolin. SP or CR is frequently coadministered with cefazolin, when cardiac and hepatic diseases are complicated with infectious disease. Therefore, the interaction of CR with cefazolin observed here is considered to be clinically significant in planning dosage regimens.

Displacement experiments showed that CR binds to site I on HSA. Fehske *et al.* (8) proposed that site I consists of two regions, known as the warfarin and azapropazone regions. Recently, we reported evidence to support Fehske's proposal (9). We showed that binding of CR in the presence of warfarin and vice versa were competitive. Moreover, interactions of CR-phenylbutazone or phenylbutazone-CR indicated that the two ligands interacted anti-cooperatively. This suggests that CR does not bind to the azapropazone region but to the warfarin region in site I on HSA. The binding of CR was almost independent of site II binding. These results suggest that CR can interact with site I-binding drugs including warfarin and cefazolin. However, the interaction of SP or CR with site I drugs, including warfarin, was not generated in serum except for the CR-cefazolin system (Fig. 3). The differences in displacement data shown in Fig. 2 and Fig. 4 can be due to differences in protein constitution as well as differences in concentrations of drugs and albumin. Serum contains the two measure binding proteins, albumin and AGP, and the low drug-to-albumin ratios were used in comparing serum with the isolated albumin system.

SP was found to markedly displace quinaldine red, a marker of a basic drug binding site on AGP (data not shown), implying the possibility of displacement of SP by basic drugs. In fact, various basic drugs induced the displacement of SP bound to serum protein with increased concentrations of the drugs. Since AGP levels in serum are increased in several disease states such as inflammation and renal insufficiency,

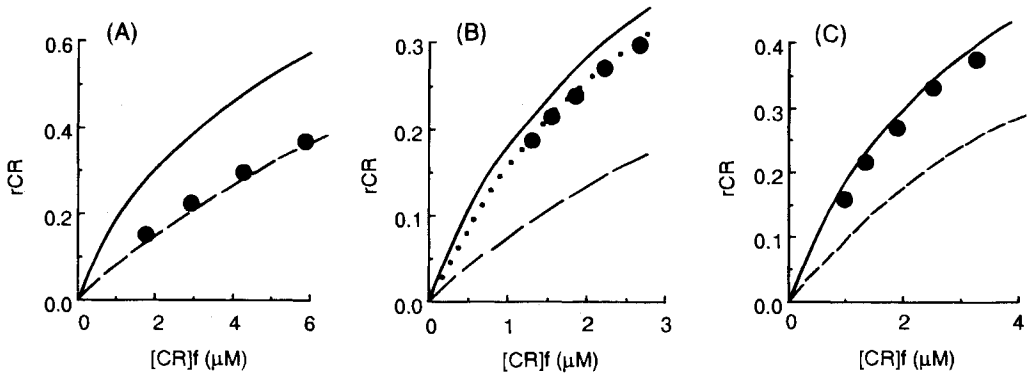


Fig. 4. Binding of CR to HSA in the presence of warfarin (A), phenylbutazone (B) and DNSS (C) at pH 7.4 and 25°C. (A) Binding of CR (20–50 μM) to HSA (120 μM) in the presence of warfarin (80 μM). (B) Binding of CR (30–50 μM) to HSA (120 μM) in the presence of phenylbutazone (60 μM). (C) Binding of CR (20–40 μM) to HSA (120 μM) in the presence of DNSS (80 μM). ●, Experimental values; _ _ _ _, Theoretical curve assuming competitive binding; _____, Theoretical curve assuming independent binding; - - - - -, Theoretical curve assuming anti-cooperative interaction ($\chi = 0.8$). All the theoretical curves were constructed by using the n_i and K_i values given in Table 2 (for warfarin, $n_1 = 1$ and $K_1 = 2.8\times10^5$; for phenylbutazone, $n_1 = 1$ and $K_1 = 1 \times 10^6$ and for DNSS, $n_1 = 1$ and $K_1 = 8.5 \times 10^5$).

interactions of SP with basic drugs can be generated. Therefore, the results presented here will provide a useful basis for planning the treatment of renal patients.

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