Effects of Fatty Acids on Serum Binding between Furosemide and Valproic Acid

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The effects of fatty acids, including oleate, on the interaction between furosemide and valproic acid in sera at respective serum therapeutic concentration levels were investigated using an ultratitration technique. The free fraction of furosemide was significantly increased in the presence of valproic acid. Mutual displacement experiments indicated that furosemide and valproic acid share a common high affinity binding site on human serum albumin (HSA). The serum free fraction of furosemide was increased by the presence of six or more fatty acid molecules per HSA molecule. This fatty acid-induced increase in the unbound fraction of furosemide was further increased by the binding of valproic acid. However, the inhibition of furosemide binding to serum for a fatty acid–valproic acid–furosemide system is nearly the same as the additive effect of fatty acid and valproic acid on the furosemide to serum. Thus, the mechanism for the displacement of HSA-bound furosemide by valproic acid was concluded to be different from that for fatty acid-catalyzed displacement.

Key words serum binding; human serum albumin; furosemide; valproic acid; fatty acid; displacement

Loop diuretics such as furosemide are frequently coadministered with other drugs for the treatment of edema. For example, furosemide is coadministered with valproic acid to relieve edema in patients suffering from petit mal and asthma. Both of these drugs bind strongly to albumin. Recently, we found that loop diuretics bind to site 1, particularly to the warfarin region on human serum albumin (HSA). It is also known that levels of fatty acids which modulate drug–protein interaction are significantly increased in patients. Thus, in these continuing investigations, we report the effects of fatty acids on the interaction between furosemide and valproic acid in sera.

MATERIALS AND METHODS

Materials HSA (essentially fatty acid free) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Furosemide was a gift from Hoechst Japan Co. (Tokyo, Japan). Stearic acid sodium salt was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Oleic acid sodium salt, linoleic acid sodium salt and valproic acid were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade. All solutions were prepared in deionized and distilled water. Phosphate buffer (67 mm, pH 7.4) was prepared from sodium dibasic phosphate and sodium monobasic phosphate, and was used exclusively in this study. Sera were separated from blood samples obtained from five healthy male subjects (mean age: 28.4 ± 6.3). The subjects were withdrawn from any medication for a minimum of 5 d prior to blood sampling.

Methods Ultrafiltration Method: Ultrafiltration experiments were performed using a Tosoh plastic ultrafiltration apparatus (Kanagawa, Japan). Aliquots of various ratios of drug–HSA (120 μM) or serum protein (500 μM equivalent to HSA) mixtures (0.9 or 1.35 ml) were centrifuged at 1800 g for 15 min at 25 °C. Adsorption of drugs to the membrane or the apparatus was negligible. No protein leakage was detected during the experiment. The free concentration of furosemide was determined by a previously described HPLC method. Valproic acid was assayed by an Abbot TDx (fluorescence polarization immunoassay) machine. The coefficients of variation of these assays were less than 5% for all ligands. The statistical significance of binding data was evaluated using one-way analysis of variance (ANOVA).

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

\[ r = \frac{[D_0]}{[P]} \cdot \sum_{i=1}^{n} \frac{n_i K_i [D_i]}{1 + K_i [D_i]} \]  

where \( r \) is the number of moles of bound drug per protein molecule, \([D_0]\) and \([D_i]\) are, respectively, the bound and unbound drug concentrations, \([P]\) is the total protein concentration, and \( K_i \) and \( n_i \) are the binding constant and the number of binding sites for the ith class of binding sites, respectively. More than 20 data points were used for the Scatchard analysis. Experimental data were reasonably fitted for Scatchard analysis (AIC (Akaike’s information criterion)<10). The simultaneous binding of two ligands was analyzed using a previously reported method.

RESULTS AND DISCUSSION

The effects of valproic acid on the free fraction of furosemide to serum were examined at serum therapeutic concentration levels (furosemide: 6.6 μM/ml, valproic acid: 57.7 μM/ml) (Fig. 1). The free fraction of furosemide was significantly increased by valproic acid. The fact that furosemide and valproic acid bind exclusively to the albumin fraction in serum suggests that the two drugs share a common high affinity binding site on the HSA molecule.

Mutual displacement experiments were carried out and the data were analyzed on the basis of a theoretical model which...
assumes the simultaneous binding of two ligands. Figure 2 shows the binding of furosemide to HSA in the presence of valproic acid (Fig. 2A) and vice versa (Fig. 2B). The binding parameter for valproic acid \( (n=1.0, K=2.8 \times 10^4 \text{ M}^{-1}) \) was somewhat different from the literature value \( (n=2.06, K=2.69 \times 10^4 \text{ M}^{-1}) \). This discrepancy may be due to differences in experimental conditions, such as the albumin lot (difference in fatty acid content and mercapto albumin content), temperature and salt concentration. The binding parameter \( (n_1=1.0, K_1=2.0 \times 10^4 \text{ M}^{-1}, n_2=3.5, K_2=3.5 \times 10^{-4} \text{ M}^{-1}) \) for furosemide was almost the same as the previously reported values \( (n_1=1.0, K_1=1.9 \times 10^4 \text{ M}^{-1} \text{ and } n_2=3.5, K_2=3.0 \times 10^{-4} \text{ M}^{-1}) \). Analysis was done using high affinity binding parameters because a simple competition could be generated between furosemide and valproic acid for identical HSA binding sites. Therefore, the experiments were carried out at low drug-to-HSA ratios. In both cases, the observed data fit the theoretical curve, assuming competitive binding between furosemide and valproic acid. This result supports the hypothesis that furosemide and valproic acid bind to site I on HSA.\(^9\) We recently reported that furosemide binds to the warfarin region at site I.\(^1\) Although site I consists of three regions (warfarin, azapropazone and p-aminobenzoate regions),\(^1\) it is likely that valproic acid also bound to the warfarin region.

Free fatty acid concentration varies depending upon the diseased state. For example, the levels of fatty acid for many patients with chronic renal dysfunction, who receive heparin as an anticoagulant during hemodialysis treatment, are enhanced. Such increases in fatty acids level are often associated with the modulation of lipid binding to albumin by competitive and allosteric displacements.\(^5\) Among fatty acids, oleate is the most abundant in human serum. Additionally, linoleate and stearate are also present in significant amounts in serum. Thus, the effects of these three fatty acids on the serum protein binding of furosemide in the presence of valproic acid were examined. Figure 3 shows the effects of oleate on the free fraction of furosemide in serum with or without valproic acid. The serum free fraction of furosemide was decreased for oleate/HSA ratios of up to 4 in the absence of valproic acid, and then increased for ratios up to 6. Six or more oleate molecules bind to one HSA molecule, and oleate occupies not only high affinity sites, but also low affinity sites including site I. As a result, oleate competes with furosemide for binding to site I. This oleate-induced increase in the unbound fraction of furosemide was further promoted by the binding of valproic acid to HSA. However, the inhibition of furosemide binding to serum for the case of the oleate-valproic acid-furosemide system is nearly the same as the additive effect of oleate and valproic acid on the binding of furosemide to serum, which differs from our recently reported conclusions.\(^7\)\(^1\) In the earlier studies, the increased free fractions of drugs were explained on the basis of combined direct and cascade effects of uric acid and fatty acids. Therefore, in the case of the oleate-valproic acid-furosemide system, it is reasonable to assume that the cascade effect of valproic acid and fatty acid contributes to the enhancement of the free fraction of furosemide only mini-
mally.

Like oleate, similar effects on furosemide binding in the presence of valproic acid were qualitatively observed for both linoleate and stearate. The effect of fatty acids on the free fraction of furosemide in the presence of valproic acid is in the order of oleate>>linoleate>>stearate (data not shown). Consequently, valproic acid and fatty acid may affect the binding of furosemide to HSA in an independent manner, even though the two ligands are structurally analogous: the displacement mechanisms of HSA-bound furosemide by valproic acid and fatty acid appear to be different.

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