Inhibitory Effects of Amino-Acid Fluids on Drug Binding to Site II of Human Serum Albumin *in Vitro*

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The effects of amino-acid fluids on ligand binding to human serum albumin (HSA) were investigated by fluorescence and ultrafiltration techniques. Warfarin and dansylsarcosine were used as the site marker fluorescence probes for site I and site II of HSA, respectively. Amino-acid fluids specifically decreased the fluorescence intensity induced by dansylsarcosine-HSA binding without any effects on that induced by warfarin-HSA binding. The ultrafiltration technique clarified that the free fraction of the site II drug, diazepam, in human serum was increased in the presence of amino-acid fluids, while no effect was observed in the free fraction of the site I drug, warfarin. The potencies of the effect on binding to site II, observed by fluorescence and ultrafiltration techniques, correlated well with the L-tryptophan contents in amino-acid fluids or with those in L-tryptophan solutions. Based on the comparison between the effects of amino-acid fluids and L-tryptophan solutions, we confirmed that L-tryptophan in amino-acid fluids specifically inhibits drug binding to site II of HSA.

Key words protein binding; albumin; parenteral nutrition; binding site; amino acid; drug interaction

Human serum albumin (HSA) is a major protein component of blood plasma and plays an important role in the regulation of colloidal osmotic pressure, antioxidant capacity of human plasma, and the transport of numerous endogenous compounds such as fatty acids, hormones, toxic metabolites (*e.g.* bilirubin), bile acids, amino acids, and metals.[1,2] The protein also binds a wide variety of drugs,[1—3] which has a significant impact on the pharmacokinetics and pharmacological effects of these drugs.[4,5] Free drug concentration can be affected by the presence of other drugs or endogenous compounds, or by microenvironmental changes in disease states. Diminished drug binding is usually the result of either competitive displacement from the same binding site or allosteric displacement following microenvironmental changes at the binding site. Several drugs bind with high affinity to one of the HSA sites[4,6] and these specific binding sites have been characterized since several decades.[7—15] Sudlow *et al.*[7,8] characterized two sites for drug binding, namely site I (also referred to as the warfarin binding site) and site II (the benzodiazepine binding site). Sjöholm *et al.*[9] have suggested the existence of one more binding site (the digitoxin site). The location of the latter site is largely unknown; however, crystallographic studies have assigned the location of sites I and II to subdomains IIA and IIIA of HSA, respectively.[3,10,11] This assignment of sites is supported by binding studies with fragments of HSA.[12—15]

Since its first description by Wilmore and Dudrick in 1968,[16] parenteral nutrition has been an integral part of the medical management for a variety of patients who are in hypermetabolic states, or suffering from neurologic disease, gastrointestinal disease, cancer, and psychiatric illness.[17] This is because the nutrients required for humans; carbohydrates, fats, amino acids, electrolytes, vitamins, and trace minerals are considered to be available for use in parenteral feeding formulations.[18,19] Despite the highly successful use of parenteral nutrition for several years, some adverse events have been reported, mostly regarding the errors in management of therapy using parenteral nutrition.[20] Most of the patients in whom parenteral nutrition is used, are also administered some therapeutic drugs, either orally, parenterally, or intravenously. The interaction between these drugs and the parenteral nutrition fluids may contribute to some cases of the adverse events. However, little information is available on the drug–parenteral nutrition fluid interaction, most notably with respect to the change in protein binding of the drug.[21—25]

This study was undertaken to evaluate the effects of parenteral nutrition fluids, especially amino-acid fluids, on protein binding to drugs *in vitro*. The present findings on the interaction between amino-acid fluids and drugs were well explained based on the concept of binding sites.

MATERIALS AND METHODS

Materials HSA (fraction V, fatty acid free), warfarin, dansylsarcosine, and L-tryptophan were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{14}C]Warfarin (56 mCi/mmol) and [^{14}C]diazepam (55 mCi/mmol) were synthesized and purified by Amersham Biosciences Corp. (Piscataway, NJ, U.S.A.) and American Radiolabelled Chemical Inc. (St. Louis, MO, U.S.A.), respectively. All other chemicals were of analytical grade. The phosphate buffer (67 mM, pH 7.4) used was prepared with sodium phosphate dibasic and sodium phosphate monobasic salts. All ligand molecules were first dissolved in methanol; the final methanol concentration was less than 1% (v/v). Amino-acid fluids were obtained as follows: Aminoleban®, Kidmin®, Amiparen®, Aminofluid®, and Aminotripa® No. 1 from Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan); Proteamin 12 X Injection® and Fulcaliq® 1 from Tanabe Seiyaku Co., Ltd.

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Table 1. Amino Acid Contents in Amino-Acid Fluids Used

Amino-acid fluids[a]	Contents of amino acids (g/l)																			
	L-Arginine	L-Histidine	L-Isoleucine	L-Leucine	L-Lysine	L-Methionine	L-Phenylalanine	L-Threonine	L-Tryptophan	L-Valine	Glycine	L-Proline	L-Serine	L-Aspartic acid	L-Glutamic acid	L-Tyrosine	L-Cystine	L-Cystein	L-Alanine	Total free amino acids
A[b]	1.58	1.20	1.12	2.50	1.76	0.70	1.87	1.30	0.26	0.90	2.14	0.66	0.44	0.76	1.30	0.07	—	0.20	1.24	20.00
B[c]	2.46	1.04	1.88	2.99	1.77	0.86	1.71	1.06	0.35	1.99	1.22	1.42	0.93	0.11	0.11	0.11	—	0.22	1.90	22.15
C[b]	3.09	1.47	2.35	4.12	3.09	1.15	2.06	1.68	0.59	2.35	1.74	1.47	0.88	0.29	0.29	0.15	—	0.29	2.35	29.41
D[b]	3.15	1.50	2.40	4.20	3.15	1.17	2.10	1.71	0.60	2.40	1.77	1.50	0.90	0.30	0.30	0.15	—	0.30	2.40	30.00
E[b]	6.00	2.40	9.00	11.00	6.10	1.00	1.00	4.50	0.70	8.40	9.00	8.00	5.00	—	—	—	—	0.30	7.50	79.90
F[b]	4.50	3.50	9.00	14.00	5.05	3.00	5.00	3.50	2.50	10.00	—	3.00	3.00	1.00	1.00	0.50	—	1.00	2.50	72.05
G[b]	10.50	5.00	8.00	14.00	10.50	3.90	7.00	5.70	2.00	8.00	5.90	5.00	3.00	1.00	1.00	0.50	—	1.00	8.00	100.00
H[b]	12.23	5.30	5.97	11.38	7.87	4.33	9.74	5.04	1.87	6.90	15.68	10.63	4.67	2.02	1.02	0.57	0.23	—	8.21	113.62

a) A; PN-twin®-No. 1, B; Fulcaliq® 1, C; Aminotripa® No. 1, D; Aminofluid®, E; Aminoleban®, F; Kidmin®, G; Amiparen®, H; Proteamin 12 X Injection®. *b*) Fluids also contain electrolytes and/or carbohydrates. *c*) Fluid also contains electrolytes, carbohydrates, and trace vitamins.

(Osaka, Japan); PN-twin®-No. 1 from Ajinomoto Pharma Co., Ltd. (Tokyo, Japan) (Table 1). Human pooled serum was prepared from blood samples obtained from 5 healthy male subjects (age 29.3±5.4 years). All subjects were withdrawn from any medication for at least 5 d prior to blood sampling. Concentration of albumin was determined to be 748 μM using Roche COBAS Integra 400 plus (Roche Diagnostics, Basel, Switzerland).

Fluorescence Measurement Steady-state fluorescence measurements were made using a Shimadzu RF-5300 PC spectrofluorometer (Shimadzu Co., Kyoto, Japan) with thermostated devices. All studies were performed at 25 °C using 5 nm excitation and emission band widths. Concentrations of HSA, warfarin, and dansylsarcosine were 2 μM. Amino-acid fluids or L-tryptophan solution (0—50 μl) were added to the mixture solution (3 ml) of HSA-warfarin or HSA-dansylsarcosine. The fluorophores of warfarin and dansylsarcosine bound to HSA were excited at 320 and 350 nm, respectively. Intensities were measured at peak maximum and corrected for any dilution effects. Although protein concentrations were maintained at low levels, to minimize inner-filter effect, the intensities were further corrected using the following equation;[26]

$$F_c = F_{obs} \text{ antilog}[(A_{exc}+A_{em})/2]$$

where F_{obs} and F_c are the observed and corrected fluorescence intensities, and A_{exc} and A_{em} are the absorbances at excitation and emission wavelengths, respectively.

Ultrafiltration Ultrafiltration experiments were performed using Minicent-10 centrifugal filter devices (Tosoh Co., Tokyo, Japan). The two compartments were separated by a cellulose membrane, which had a molecular weight cut-off of 10000 Da. HSA concentration in human serum was adjusted to 500 μM by diluting the serum with phosphate buffer (pH 7.4). Subsequently, an aliquot (200 μl) of the mixture consisting of the diluted human serum and the radio-labeled probe (5 μM) was placed in the upper compartment and centrifuged at 3000 rpm for 10 min at 25 °C. The radioactivities of both solutions before filtration (RA_{before}) and after filtration (RA_{after}) were determined by liquid scintillation counting after adding scintillation fluid (Nacalai Tesque, Kyoto, Japan) to scintillation vials. The free fraction of the radio-labeled probe was calculated as a % ratio of the activities of the solutions before and after filtration using the following equation:

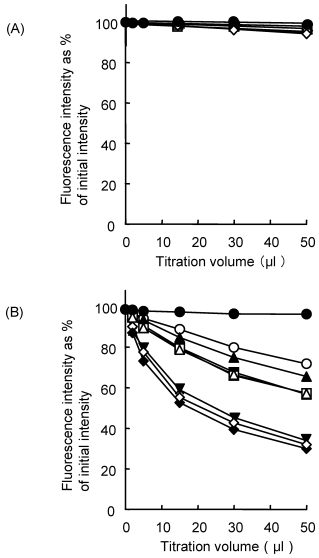

Fig. 1. Effects of Amino-Acid Fluids on the Fluorescence Induced by HSA-Warfarin (A) and HSA-Dansylsarcosine (B) Interactions

Amino-acid fluids used were PN-twin®-No. 1 (○), Fulcaliq® 1 (▲), Aminotripa® No. 1 (△), Aminofluid® (■), Aminoleban® (□), Kidmin® (◆), Amiparen® (◇), and Proteamin 12 X Injection® (▼). Normal saline solution was used as a control (●). The concentration of HSA, warfarin and dansylsarcosine was 2 μM each. Each amino-acid fluid was added to 3 ml of HSA-warfarin or HSA-dansylsarcosine solution. The data were the mean values of three experiments.

$$\text{free fraction (\%)} = RA_{after}/RA_{before} \times 100$$

The effects of amino-acid fluids or L-tryptophan solution were investigated by the addition of 2 μl of each of these to the mixture described above. The adsorption of warfarin or diazepam onto the filtration membrane and the apparatus was negligible.

RESULTS

Effects of Amino-Acid Fluids on Fluorescence of Site Marker Fluorescence Probes Bound to HSA Warfarin and dansylsarcosine were used as site marker fluorescence probes for monitoring site I and site II of HSA, respectively.[2,7,8] The effects of amino-acid fluids on the enhanced fluorescence of each probe, which is induced by their bindings to HSA, were investigated. As shown in Fig. 1, the fluorescence intensity of the warfarin–HSA complex was not in-

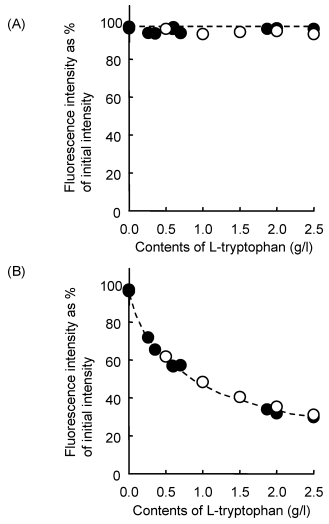

Fig. 2. Correlation between Fluorescence Intensity (% of Initial Intensity) in the Presence of Amino-Acid Fluids (---●---) or L-Tryptophan Solutions (○) and Their L-Tryptophan Contents

50 μl of amino-acid fluids or L-tryptophan solutions was added to 3 ml of HSA-warfarin (A) or HSA-dansylsarcosine (B) solution. Contents of L-tryptophan in amino-acid fluids are indicated in Table 1 and those in L-tryptophan solutions were 0, 0.5, 1.0, 1.5, 2.0, and 2.5 g/l. L-Tryptophan solutions were prepared with nomal saline solution. The concentration of HSA, warfarin, and dansylsarcosine was 2 μM each. The data were the mean values of three experiments.

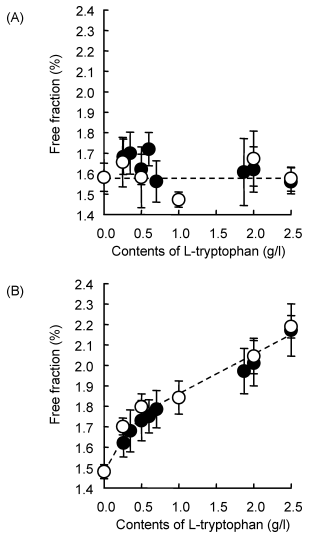

Fig. 3. Effects of Amino-Acid Fluids (---●---) or L-Tryptophan Solutions (○) on the Free Fraction of [^{14}C]Warfarin (A) and [^{14}C]Diazepam (B) in Human Serum

Normal saline solution was used as a control (L-tryptophan contents; 0 g/l). The concentration of HSA in human serum was adjusted to 500 μM, those of [^{14}C]warfarin and [^{14}C]diazepam were 5 μM. 2 μl of amino-acid fluids were added to 200 μl of [^{14}C]warfarin- or [^{14}C]diazepam-human plasma mixture solution. Results are presented as the mean±S.D. of three independent experiments.

fluenced by any amino-acid fluids even when larger volumes were added (volume of 50 μl). However, amino-acid fluids decreased the fluorescence intensity of dansylsarcosine–HSA complex in a fluid volume-dependent manner. Among the amino acids included in each fluid, L-tryptophan contents correlated well with the extent of the fluid's effects on the dansylsarcosine–HSA interaction (Fig. 2B, closed symbols and line). The effects of L-tryptophan solutions were also examined to confirm whether L-tryptophan is responsible for these effects. The plot of the effects (Fig. 2B, open symbols) coincides well with the correlation curve described by the effects of amino-acid fluids. Thus, it was concluded that L-tryptophan in amino acid fluids plays a major role in the change in dansylsarcosine–HSA interaction. The other fluids examined (fluids containing electrolytes, carbohydrates, and/or trace vitamins) did not show any effects both on warfarin-HSA and on dansylsarcosine–HSA interactions (data not shown).

Effects of Amino-Acid Fluids on Bindings of Drugs to HSA in Human Serum The binding of warfarin and diazepam to HSA in human serum was examined using ultrafiltration techniques. Warfarin and diazepam are drugs that bind specifically to site I and site II of HSA, respectively.[1,2] As shown in Fig. 3, amino-acid fluids and L-tryptophan solution selectively increased the free fraction of diazepam without any effect on warfarin binding. The potency of the effects on diazepam binding correlated well with the L-tryptophan contents in the amino-acid fluids (Fig. 3B, closed symbols and line) and in the L-tryptophan solution (Fig. 3B, open symbols). These results indicated that L-tryptophan in the amino-acid fluids inhibits the drug binding to site II of HSA in human serum.

DISCUSSION

Parenteral nutrition is now widely used for the treatment of malnutrition.[18—20] Among the components of parenteral nutrition, fluids including amino acids, electrolytes, carbohydrates, and/or trace vitamins (Table 1) were investigated with respect to their effects on protein binding.

The fluorescence technique, which is a useful tool for rapid screening of the factors that affect the ligand interaction with each binding site of HSA,[7,8,27,28] showed that amino-acid fluids had specific effects on only the interaction of dansylsarcosine with site II of HSA without any effects on the interaction of warfarin with site I (Fig. 1). Although amino-acid fluids also contain electrolytes, carbohydrates and/or trace vitamins, fluids containing electrolytes, carbohydrates, or trace vitamins did not show any effects (data not shown). Thus, amino acid(s) contained in fluids might affect the ligand interaction with site II of HSA.

Site II specific effects were also observed in drug binding to human serum. The ultrafiltration results indicated that the amino-acid fluids specifically displaced the site II drug, diazepam, without any effects on the binding of the site I drug, warfarin (Fig. 3). Since warfarin and diazepam specifically bind to HSA in human serum,[29—32] these findings could be explained by the specific inhibition of the binding to site II of HSA, which is consistent with the results of the fluorescence technique. Furthermore, the site II specific effects of amino-acid fluids, observed by both fluorescence and ultrafiltration techniques, were confirmed to be only due to the L-tryptophan contents in amino-acid fluids. This was based on the comparison of the results between the effects of amino-acid fluids and those of L-tryptophan solutions. Plots demonstrating the effects of L-tryptophan solutions fit the correlation curve of the effects between amino-acid fluids and their L-tryptophan contents (Figs. 2, 3).

L-Tryptophan has been known to be the only amino acid that binds to one high affinity binding site of HSA, site II, with an affinity constant of $1—6.2\times10^4\,M^{-1}$ at 25—37 °C.[33—35] Kragh-Hansen[36] reported that diazepam and L-tryptophan compete for a common high affinity binding site on HSA. Therefore, our present findings suggest that the reduction in binding of diazepam to site II by amino-acid fluids is due to the competitive displacement by L-tryptophan in the fluids. The affinity constant of L-tryptophan is lower than that of commonly used site II drugs ($10^5—10^6\,M^{-1}$).[1,2] The increased concentration of amino acid upon infusion of amino-acid fluid was reported to be closely correlated to their infusion rate in healthy volunteers (increased concentration$=1.46\times$infusion rate-18.27, $r^2=0.92$).[37] According to this correlation, the concentration of L-tryptophan might increase by 12 μM (for Kidmin®) higher than the concentration prior to infusion (about 40 μM), whereas, in our experiments, the final concentrations of increased L-tryptophan in human serum are 13 μM (for PN-twin-No. 1®)—122 μM (for Kidmin®). Thus, *in vivo* studies on the effect of co-administered amino-acid fluids on drug binding to site II should be undertaken in the future. This may be especially important in patients with certain disease states in which albumin concentration is decreased (*e.g.* malnutrition, liver dysfunction, nephrosis, GI losses, shock, edema).[1] Monitoring of free drug concentrations may be useful for these and other patients in whom parenteral nutrition with amino-acid fluids is used, particularly for those drugs that are bound to site II on HSA.

In conclusion, L-tryptophan in amino-acid fluids competitively displaces the drug bound to site II on HSA. The present findings are very useful for evaluating the pharmacokinetics and pharmacological effects of therapeutic drugs in patients on parenteral nutrition with amino-acid fluids.

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