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AURAMINE O AS A FLUORESCENT PROBE FOR THE BINDING OF BASIC DRUGS TO HUMAN α_1-ACID GLYCOPROTEIN (α_1-AG)

THE DEVELOPMENT OF A SIMPLE FLUOROMETRIC METHOD FOR THE DETERMINATION OF α_1-AG IN HUMAN SERUM

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Abstract—A cationic fluorescent dye, auramine O (AO), exhibited an intense increase in fluorescence after binding to human α_1-acid glycoprotein (α_1-AG). The interaction between AO and the protein was studied by fluorescence spectroscopy and by equilibrium dialysis. AO binds to the protein via a single site with a dissociation constant of 24 μM. Various basic drugs such as chlorpromazine, imipramine, desipramine, quinidine, propranolol and lidocaine, which are known to bind to the protein, competitively inhibited the AO binding to the protein. The dissociation constants of these basic drugs obtained from such inhibitory experiments were comparable to those obtained with other methods (equilibrium dialysis, quenching of protein intrinsic fluorescence, and the difference spectrophotometric method) and from the literature. It is concluded that AO may be a useful fluorescent probe that binds to a single basic drug binding site on α_1-AG. In addition, a simple fluorometric method for the determination of α_1-AG in serum was developed using AO, and the validity of this method was confirmed by comparing it with the conventional radial immunodiffusion method.

While albumin is the major binding protein for acidic drugs, increasing evidence that α_1-acid glycoprotein (α_1-AG) also plays an important role in the binding of various basic drugs in serum [1–4] has been accumulating during recent years. α_1-AG received much attention in the interpretation of pharmacokinetic data of basic drugs [1, 5]. It has been reported that the serum α_1-AG level increases in patients with cancer [6] and arthritis [7] and following myocardial infarction [8] and surgery [9]. Thus, large variations in the serum level of α_1-AG observed in these patients could also have accounted for similarly large variations in the unbound serum levels of some basic drugs [1, 2, 10, 11] which have been believed to be responsible for the pharmacological effect. Therefore, the importance of measuring α_1-AG concentration in serum of individual patients has been stressed [1]. Radial immunodiffusion using antibody against α_1-AG has been widely used to determine α_1-AG concentration in serum [2, 7, 10, 11]. This method is time-consuming, although it has an advantage with respect to specificity.

Only a few observations are available about the molecular aspects of the interaction of drugs with α_1-AG [4, 12]. This is in contrast to an abundance of information about the interactions of acidic drugs with serum albumin. That is, the nature of the binding sites of various acidic drugs on serum albumin, of the displacement by other drugs and some endogenous substances like free fatty acids, and of the alteration of serum protein binding in several disease states is well understood [13, 14].

The discovery and proper usage of the fluorescent probe have provided useful information for the understanding of the interaction between proteins and small molecules [15–17]. For instance, 1-anilino-8-naphthalenesulfonate (ANS), dansylamide and other fluorescent dyes have been used extensively in the characterization of drug binding sites on serum albumin [18–20].

In the present study, we describe the development of a rapid and simple fluorometric method for measuring the serum concentration of α_1-AG by use of a fluorescent dye, auramine O (AO), and, in addition, examine whether this fluorescent dye, AO, and various basic drugs utilize common or separate binding sites on α_1-AG.

MATERIALS AND METHODS

Materials

Serum samples were obtained from fourteen healthy and drug-free male subjects (22- to 52-years-old). Venous blood was sampled and was allowed to stand for 1 hr at room temperature. Serum was then separated by centrifugation at 1500 *g* for 15 min and was stored at −40° until use. Commercial human α_1-AG, human serum albumin (HSA), and γ-globulin were supplied by the Sigma Chemical Co. (St. Louis,

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MO). The concentration of α_1-AG was determined by use of the reported extinction coefficient, $E^{1\%}_{278nm} = 8.93$ [21]. The molecular weight of α_1-AG was assumed to be 44,100 [22]. In serum samples, α_1-AG was determined by radial immunodiffusion on Partigen M plates (Hoechst Japan, Tokyo, Japan) and by the fluorometric method developed in the present study. Auramine O (AO) was obtained from the Eastman Kodak Co. (Rochester, NY) and was recrystallized gently from 0.02 M NaCl. The temperature was kept below 30° to avoid hydrolysis [23]. Sulfosalicylic acid (SSA) was obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Chlorpromazine (CPZ) and imipramine (IPM) were supplied by Yoshitomi Pharm. Ind. Co. Ltd. (Osaka, Japan). Desipramine (DPM) was supplied by Ciba-Geigy Japan Co. Ltd. (Tokyo, Japan). Quinidine (QD) and acridine orange-10-dodecyl bromide (AODB) were purchased from Wako Pure Chemical Ind. Ltd. Propranolol (PL) was purchased from Sumitomo Chemical Co. Ltd. (Osaka, Japan), and lidocaine (LC) from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Diazepam (DZP) and phenobarbital (PB) were supplied by the Yamanouchi Pharm. Co. (Tokyo, Japan) and the Sankyo Pharm. Co. (Tokyo, Japan) respectively. Salicylic acid (SA) was purchased from the Koso Chemical Co. (Tokyo, Japan). Tolbutamide (TB) and warfarin (WF) were supplied by Hoechst Japan and Eizai Co. Ltd, (Tokyo, Japan) respectively. Progesterone and hemoglobin were purchased from the Sigma Chemical Co. [^{3}H]Desipramine (67.8 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA) and found to be more than 98% pure by thin-layer chromatography. Bilirubin was purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan). All other chemicals used were commercially available and of analytical grade.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn [24]. Prior to the electrophoresis, the protein samples were incubated with 0.9% mercaptoethanol for 10 min at 85°.

Equilibrium dialysis

Equilibrium dialysis was performed at 37° using dialysis cells as reported previously [25]. All solutions were prepared in 0.13 M potassium phosphate buffer, pH 7.4 (standard buffer). After equilibration was attained at 7 hr, the concentrations of the drug in both chambers, i.e. protein and buffer sides, were determined. The concentration of AO was determined spectrophotometrically ($\lambda_1 = 550$ nm; $\lambda_2 = 430$ nm) in a Hitachi 557 double wavelength double beam spectrophotometer (Hitachi Ltd., Tokyo, Japan). The concentrations of DPM and QD were determined by liquid scintillation spectrometry and by the fluorometric method [26] respectively. The binding data were fitted to a Langmuir-type equation by an iterative non-linear least squares method. The initial parameters were obtained from Scatchard-type plots.

Determination of dissociation constant for AO in the presence and absence of inhibitors by fluorometric titration

Binding studies for AO were performed at room temperature (23–26°) in a Hitachi MPF-4 fluorospectrometer (Hitachi Ltd.). To the cuvettes containing 2 ml of 4.3 μM α_1-AG in the standard buffer with or without inhibitors were added small aliquots (2–6 μl) of AO stock solution (5 mM in ethanol), up to 30 μl of the total sample volume. The solution was stirred with a Pasteur pipette after each addition, and the fluorescence intensity was measured at 550 nm (excited at 490 nm). If necessary, the fluorescence intensity was corrected for the inner filter effect by the method of Chignell [15]. The apparent dissociation constant ($K_{d,app}$) was calculated from modified Scatchard-type plot [10, 11], namely the plot of the change in fluorescence (ΔF)/AO concentration against ΔF.

Determination of dissociation constants for various basic drugs by the fluorescence change of AO

Inhibitory binding studies were performed at room temperature by following the decrease in the fluorescence of AO. To cuvettes containing 2 ml of a mixture of 4.3 μM α_1-AG and 40 μM AO in the standard buffer we added a maximum of 30 μl of competitors in 2.6 μl aliquots. The fluorescence intensity was measured after each addition as described above. The dissociation constant (K_I) for the inhibitor was calculated from such competitive studies as described previously [27]. In brief, the inhibition data [inhibitor concentration (I_t) versus fluorescence intensity (F)] were fitted to the following equation:

$$I_t = \left\{nP - \frac{F}{\psi} - \frac{F \cdot K_d}{\psi C_t - F}\right\} \times \left\{1 + \frac{K_I(\psi C_t - F)}{F \cdot K_d}\right\} \quad (1)$$

where C_t and I_t are the total concentrations of AO and the inhibitor, respectively, P is the protein concentration, ψ is the fluorescence quantum yield of bound AO, F is the fluorescence intensity of bound AO, and K_d and K_I are the dissociation constants of AO and the inhibitor respectively. This equation can be derived from the following two equations [27, 28]:

$$I_t = \left\{nP - C_b - \frac{C_b \cdot K_d}{(C_t - C_b)}\right\} \times \left\{1 + \frac{K_I(C_t - C_b)}{C_b \cdot K_d}\right\} \quad (2)$$

$$F = \psi \cdot C_b \quad (3)$$

where C_b is the concentration of bound AO. Equation 2 holds assuming two ligands, AO and a competitor, compete for a single class of n binding sites on the protein [28].

Determination of binding parameters for CPZ binding to α_1-AG by ultraviolet difference spectrophotometry

Difference absorption spectra were recorded in a Hitachi 557 double wavelength double beam spectrophotometer at room temperature. Three-milliliter solutions of α_1-AG (2.9 or 19 μM) in the standard buffer and 3 ml of the standard buffer only were

placed in each of the sample and reference cells respectively. After the baseline was corrected, the stock solution of CPZ (2 or 10 mM) was added to both cells in small aliquots (2–6 μl), and the difference spectra were recorded after each addition. Difference absorbances at 261 nm at the concentration of 2.9 μM α_1-AG were used for the calculation of binding parameters, K_d and number of binding sites. Calculation of the concentrations of unbound and bound CPZ was performed according to the method of Huang and Gabay [29].

Determination of binding parameters for CPZ binding to α_1-AG by the quenching of intrinsic fluorescence of protein

The decrease in the intrinsic fluorescence of α_1-AG upon addition of CPZ was measured at 330 nm while excited at 290 nm. The binding parameters were determined from fluorescence data obtained at two widely different protein concentrations (2 and 10 μM in the present study) according to the method of Halfman and Nishida [30]. Titrations of protein with CPZ were performed as described above.

Calculation of dissociation constants from literature data for comparison with those obtained by the AO method

Direct method. When the bindings of drugs to purified α_1-AG were measured by a direct method like equilibrium dialysis, the values of K_d obtained from the literature were used for comparison.

Indirect method. Most studies on drug binding to α_1-AG deal with the correlation between the ratio of the bound and unbound drug concentrations in serum (C_b/C_f) and the serum α_1-AG concentration. If we assume that drug binding is linear under the conditions studied and that the interindividual difference in the serum levels of other binders such as albumin and lipoprotein is small and, therefore, the interindividual difference in the serum binding of the drug is due mainly to that of serum α_1-AG concentration, the following equation can be derived:

$$\frac{C_b}{C_f} = \frac{n}{K_d} 3(\text{concentration of } \alpha_1\text{-AG}) + \text{constant} \quad (4)$$

Consequently, K_d can be calculated from the slope of the plot of C_b/C_f vs concentration of α_1-AG assuming that the binding stoichiometry (n) equals one.

Procedures for the determination of α_1-AG concentration in serum by the AO method

The following stock solutions were used: (A) 0.13 M potassium phosphate buffer, pH 7.4 (standard buffer); (B) human α_1-AG solution (5 mg/ml) in the standard buffer; (C) 2 N sodium hydroxide; (D) 6% (w/v) SSA solution; (E) 5 mM AO solution dissolved in ethanol, and (F) a mixture of 100 ml of A and 1.65 ml of C, freshly prepared before use.

The established procedures are as follows: (1) Put 0–200 μl of α_1-AG solution (solution B) into a microcentrifuge tube. (2) Add the appropriate volume of standard buffer (solution A) to get a final volume of 200 μl, and agitate on a Vortex mixer. In the measurement of serum sample, put 200 μl of serum into a centrifuge tube instead of the α_1-AG solution and the buffer. (3) Add 200 μl of SSA solution (solution D) and agitate immediately on a Vortex mixer. (4) Stand them on ice for 3 min. (5) Centrifuge for 3 min in a table top microfuge (Centrifuge B, Beckman Instruments, Fullerton, CA). (6) Take out 300 μl of each supernatant fraction and put it into a test tube containing 2 ml of solution E and mix well. (7) Put 2.2 ml of the mixture into a cuvette for the fluorescence measurement and measure the blank fluorescence (F_1) at 550 nm (excited at 470 nm). (8) Add 40 μl of AO solution (solution E) into the cuvette by use of a microsyringe and mix the solution carefully with a Pasteur pipette. Measure the fluorescence (F_2). All through the fluorescence measurement, 10 μM AO solution in 50% (v/v) glyc-

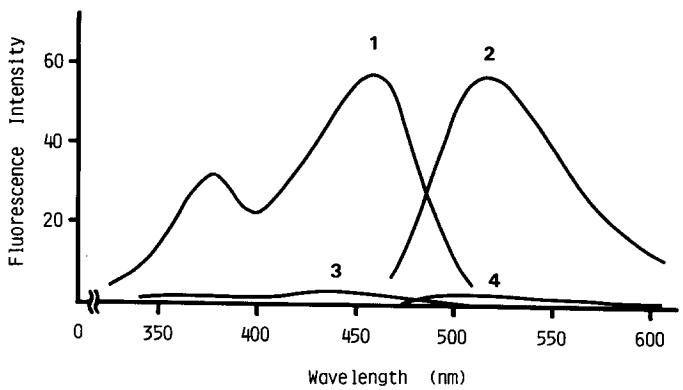

Fig. 1. Fluorescence excitation and emission spectra of auramine O (AO) in the presence and absence of α_1-AG. In the presence of α_1-AG (curve 1: excitation spectrum, and curve 2: emission spectrum), the cuvette contained 20 μM α_1-AG and 20 μM AO in 0.13 M potassium phosphate buffer (pH 7.4), while in the absence of α_1-AG (curve 3: excitation spectrum, and curve 4: emission spectrum) the cuvette contained 20 μM AO only in the same buffer. The spectra were not corrected for light source and detector non-linearity. Emission was at 520 nm for excitation spectra and excitation was at 450 nm for emission spectra.

Table 1. Dissociation constants for the binding of auramine O (AO) to α_1-acid glycoprotein (α_1-AG) and human serum albumin (HSA)*

Protein	K_d (μM)	
	Fluorometric titration†	Equilibrium dialysis‡
α_1-AG	24 ± 4	22§
HSA	120 ± 32	140‖

* Determined at 37°.
† Calculated according to the method of Wang and Edelman [16]. Values are expressed as mean ± S.E. (N = 3).
‡ Average of two independent experiments.
§ Binding stoichiometry (n) of 0.79 was obtained.
‖ Expressed as K_d/n value, since saturable binding was not obtained under the condition used.

erol was used as the fluorescence standard. (9) Obtain standard curve by plotting ($F_2 - F_1$) versus α_1-AG concentration in 200 μl of the initial solution (namely in procedures 1 and 2). In procedure 6, sodium hydroxide was added to neutralize the solution.

RESULTS

Binding of AO to α_1-AG

The fluorescence excitation and emission spectra of AO in the presence and absence of α_1-AG are shown in Fig. 1. This dye does not fluoresce in aqueous solution. In the presence of α_1-AG, there was a remarkable enhancement of dye fluorescence with excitation and emission maximum at 460 and 515 nm respectively. To determine whether the fluorescence enhancement of AO in the presence of α_1-AG was actually due to the binding to α_1-AG, $K_{d,app}$ obtained from the fluorometric titration of α_1-AG with AO was compared to that obtained by the equilibrium dialysis method. The results are summarized in Table 1 with those for HSA. The values of K_d obtained from both methods were comparable, suggesting that the fluorescence enhancement of AO by α_1-AG came from AO binding to α_1-AG. The affinity for α_1-AG was approximately five times that for HSA. The stoichiometry of AO binding to α_1-AG was determined by equilibrium dialysis (not shown in figure), and the value of 0.79 mole of AO bound per mole of α_1-AG was obtained. α_1-AG was thus considered to have only one binding site for AO.

Inhibition of AO binding to α_1-AG by various basic drugs

Figure 2 shows the inhibition of the AO binding to α_1-AG by addition of various basic drugs. CPZ was the most potent inhibitor and DPM was the least potent inhibitor among the drugs tested. On the other hand, such inhibition was not obtained in the AO binding to HSA. Figure 3 shows that IMP inhibited competitively the AO binding to α_1-AG as shown in a modified Scatchard-type plot. As the concentration of IMP increased, $K_{d,app}$ for AO also increased without change of the maximum fluorescence intensity. In addition, as shown in the insert of Fig. 3, the relation between the change in $K_{d,app}$ and the concentration of IMP fit well with an equation

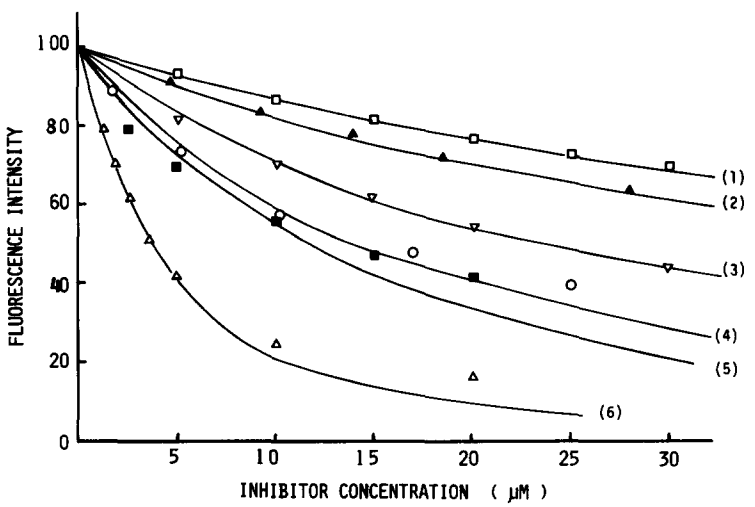

Fig. 2. Inhibition of auramine O (AO) binding to α_1-AG by addition of various basic drugs. The decrease in fluorescence intensities was measured at constant concentrations of α_1-AG (4.3 μM) and AO (40 μM) (see the text for details). The mean data of three experiments are shown. Variance of the data are 2–7%. Inhibitors used were: desipramine (DPM) (□), lidocaine (LC) (▲), imipramine (IMP) (△), propranolol (PL) (○), quinidine (QD) (■) and chlorpromazine (CPZ) (△). The solid lines are theoretical curves calculated as described previously [27], based on the assumption that these inhibitors competitively inhibit the AO binding to α_1-AG. The binding parameters used in this calculation were: K_d (dissociation constant for AO) = 24 μM, n (number of binding sites for AO) = 1 and K_I (dissociation constant for inhibitors) = (1) 23 μM, (2) 16 μM, (3) 8 μM, (4) 4.5 μM, (5) 3.5 μM, and (6) 0.6 μM.

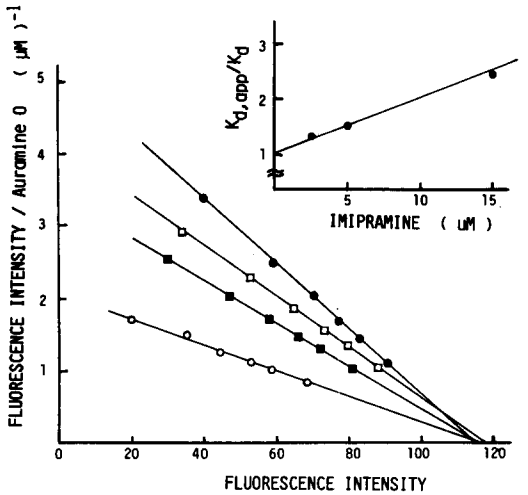

Fig. 3. Modified Scatchard plot of AO binding to α_1-AG in the absence (●) and presence of imipramine (IMP). Concentrations of IMP were 2.5 μM (□), 5 μM (■) and 15 μM (○). AO concentrations were changed from 11 to 75 μM, while the concentration of α_1-AG was kept constant (4.3 μM). The mean data of three experiments are shown. Variance of the data are 3–7%. The inset shows a relation between the ratio of dissociation constants ($K_{d,app}/K_d$) and IMP concentration (I), where K_d and $K_{d,app}$ are the dissociation constants obtained from binding experiments in the absence and presence of IMP respectively. The data were fitted to an equation: $K_{d,app}/K_d = 1 + (I)/K_I$, representing a competitive inhibition. The mean ± S.D. of the y intercept was 1.02 ± 0.02, which was close to 1.

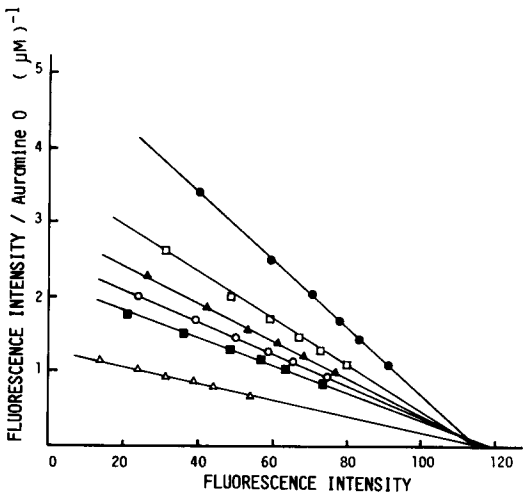

Fig. 4. Modified Scatchard plot of the AO binding to α_1-AG in the absence (●) and presence of various basic drugs. The drugs and their concentrations used were: desipramine (DPM) 10 μM (□), lidocaine (LC) 10 μM, (▲), propranolol (PL) 5 μM (○), quinidine (QD) 5 μM (■) and chlorpromazine (CPZ) (△) 5 μM. AO concentration was changed from 11 to 75 μM, while the concentration of α_1-AG was kept constant (4.3 μM). The mean data of three experiments are shown. Variance of the data are 3–9%.

Table 2. Dissociation constants for the binding of various compounds to α_1-AG

	K_d (μM)			
	From the present study		From the literature*	
Drugs	AO method†	Other methods	Direct method‡	Indirect method§
Chlorpromazine (CPZ)	0.6	0.57‖ 0.75¶	0.29 [4] 0.35 [31] 1.1 [32]	1 [7]
Quinidine (QD)	3.5	5 (high affinity site)‡ 30 (low affinity site)‡		14 [33]
Propranolol (PL)	4.5	ND**	0.88 [4] 30 [3]	3.5–5 [7, 34, 35]
Imipramine (IPM)	8	ND	4.1 [4] 11 [36]	
Desipramine (DPM)	23	25‡	21 [36]	
Lidocaine (LC)	16	ND		9–11 [11, 34, 37, 38]
Auramine O (AO)	24††	22		
Acridine orange doedecyl bromide (AODB)		0.25††		

* The number of brackets after each value shows the reference number.
† Calculated from the data shown in Fig. 2.
‡ Determined by equilibrium dialysis.
§ The method for calculation of dissociation constants (K_d) from the data in the literature is described in the text.
‖ Determined by the method of quenching of intrinsic fluorescence of α_1-AG (see Fig. 6).
¶ Determined by the difference spectrophotometric method (see Fig. 6).
** Not determined.
†† Determined by the fluorescence chance of dye after bound to α_1-AG.

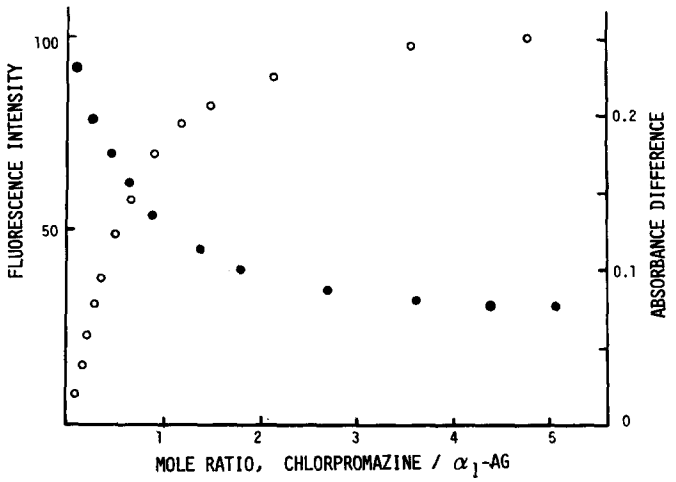

Fig. 5. Fluorometric and spectrophotometric titrations of α_1-AG with chlorpromazine (CPZ). The intrinsic fluorescence intensity (●) of α_1-AG (10 μM) was monitored at 330 nm, while excited at 290 nm. The difference ultraviolet absorbance (○) of CPZ at 261 nm was also monitored, where α_1-AG concentration was kept constant (19 μM) (see the text for details). The mean data of two experiments are shown.

(see legend for Fig. 3) representing a so-called competitive inhibition. Figure 4 shows that other basic drugs such as DPM, LC, PL, QD and CPZ also inhibited competitively the AO binding to α_1-AG, although the degree of inhibition differed among drugs. The dissociation constants for the binding of various basic drugs to α_1-AG were estimated from such inhibitory experiments based on the assumption of competitive inhibition (Fig. 2) and are listed in Table 2.

To compare the dissociation constants thus obtained with those determined by other methods, the binding of DPM and QD was measured by equilibrium dialysis and that of CPZ by the ultraviolet difference spectrophotometric method and the quenching of intrinsic protein fluorescence method. The Scatchard plot of the DPM binding shows a straight line, while the curvilinear Scatchard plot of QD binding indicates the presence of high and low affinity sites (not shown in figure). The values of K_d determined by equlibrium dialysis were 25 μM for DPM and 5 μM (the high affinity site) and 30 μM (the low affinity site) for QD.

The difference absorption spectrum for the binding of CPZ and α_1-AG was characterized by two positive (261 and 335 nm) and two negative (246 and 296 nm) absorption peaks. The peak at 261 nm shows the highest intensity, and a representative titration curve obtained at 261 nm is shown in Fig. 5. The intrinsic fluorescence of α_1-AG was also quenched by the binding of CPZ. As shown in Fig. 5, the shape of titration curve obtained by monitoring the increase in the difference absorption was similar to that obtained by measuring the quenching of the intrinsic fluorescence of α_1-AG. These titration curves suggest that both phenomena resulted from the same drug–protein interaction. Both titration curves obtained by use of the lower protein concentration (see Materials and Methods) were analyzed according to a Scatchard equation [39]. As shown in Fig. 6, the dissociation constants obtained by the difference spectrophotometric method and the fluorescence quenching method were 0.75 and 0.57 μM, respectively, which are comparable to that (0.6 μM) obtained by the AO method. The number of binding sites (n) was close to unity with both methods. Table 2 summarizes the dissociation constants thus obtained and those from the literature. The dis-

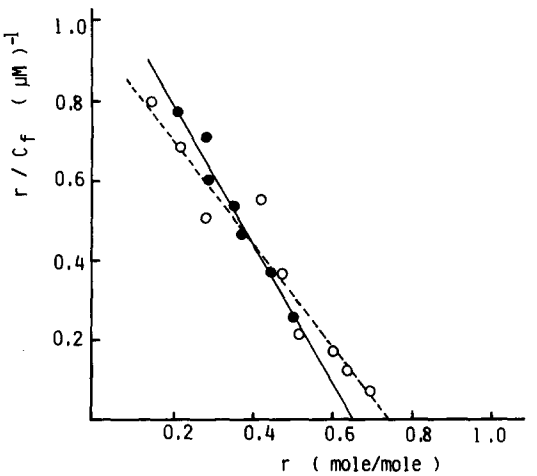

Fig. 6. Scatchard plot of chlorpromazine (CPZ) binding to α_1-AG determined by the quenching of intrinsic fluorescence (●) of α_1-AG and the change in difference ultraviolet absorbance (○) of CPZ (see the text for details). The mean data of two experiments are shown. The solid line (—●—) and dotted line (--●--) were fitted by a non-linear least squares method. Binding parameters, $n = 0.65$ and $K_d = 0.57$ μM, were obtained for the method of quenching of intrinsic fluorescence, and $n = 0.73$ and $K_d = 0.75$ μM were obtained for the method of change in the difference absorbance.

sociation constants obtained for various basic drugs by the AO method are comparable with those determined by other methods and those obtained from the literature.

Determination of α_1-AG in serum by the use of AO

Although AO had higher affinity for α_1-AG than for albumin (Table 1), the direct measurement of serum fluorescence in the presence of an excess amount of AO was not successful in determining the α_1-AG concentration in human serum. This is because the serum concentration of albumin (50 mg/ml) is much higher than that of α_1-AG (0.5 to 1 mg/ml), and the fluorescence which came from the albumin–AO complex could not be neglected. Therefore, removal of major proteins (albumin and γ-globulin) from serum was attempted by use of SSA. SSA was used previously to prepare crude α_1-AG from human serum by Routledge *et al.* [11]. Treatment of human serum with SSA removed almost all the proteins except for α_1-AG in human serum. This was confirmed as follows. First, in the SDS-gel electrophoresis, the SSA-treated human serum showed a single band corresponding to commercially available purified α_1-AG. Second, after the treatment of α_1-AG (1 mg/ml), HSA (50 mg/ml) and γ-globulin (10 mg/ml), respectively, with SSA, each fluorescence intensity was measured in the presence of an excess amount of AO (90 μM) and was compared to that obtained using the untreated protein sample. The fluorescence intensity of AO in HSA or γ-globulin was negligible after the treatment with SSA, while that in α_1-AG changed little before and after the treatment with SSA. The principle of the assay for α_1-AG established in the present study is as follows. Most of the serum proteins except for α_1-AG were removed by treatment with SSA, followed by centrifugation. Subsequently, α_1-AG left in the supernatant fraction was determined by measuring the fluorescence intensity after addition of an excess amount of AO (90 μM). A typical standard curve obtained according to the procedure described in Materials and Methods is shown in Fig. 7A. A linear relation between the α_1-AG concentration (in 0.2 ml of the initial buffer) and the fluorescence intensity was observed over the concentration range of 0.2 to 5 mg/ml α_1-AG.

By use of the newly established AO method and the conventional radial immunodiffusion method, the α_1-AG concentrations in the serum of fourteen healthy volunteers were determined. As shown in Fig. 7B, a good correlation was observed between the two methods, although the AO method had a tendency to underestimate α_1-AG concentration. The difference between the two methods was, at most, 20%. The intra-assay CV (within day variation) and the interassay CV (between day variation) of the AO method were 3.7% (N = 6) and 6.6% (N = 5), respectively, determined using a serum containing 0.83 mg/ml α_1-AG.

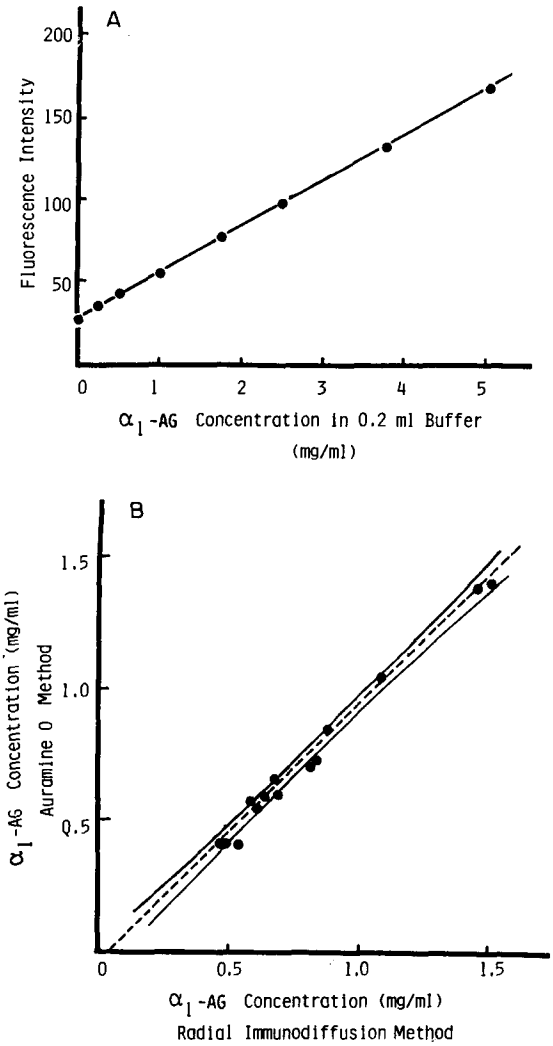

Fig. 7. (A) Standard curve for the measurement of α_1-AG by the auramine O method. The regression line is $y = 27.4x + 27.8$ ($r = 1.000$, $P < 0.01$). (B) Relation between the α_1-AG concentrations in human serum from fourteen healthy subjects determined by the radial immunodiffusion method (abscissa) and by the AO method (ordinate). The regression line shown by a dotted line is $y = 0.987x - 0.0772$ ($r = 0.993$, $P < 0.01$). The 95% confidence limits are shown by the solid lines.

DISCUSSION

We at first attempted to find cationic fluorescent dyes, which bind relatively specifically to α_1-AG. In the screening of fluorescent dyes, we took into consideration that the increase in the fluorescence quantum yield of dyes was great in the binding to α_1-AG and that the binding of dye was relatively specific for α_1-AG. After screening several dyes, we found that AO and AODB bound to α_1-AG with high affinity. AO and AODB were used previously to characterize the coenzyme and substrate binding sites of liver alcohol dehydrogenase [23] and to detect the critical micellar concentration of anionic detergent [40] respectively. As shown in Table 1, the K_d of AO for α_1-AG (24 μM) was approximately one-fifth that for human serum albumin (120 μM). In addition, a remarkable enhancement of fluorescence quantum yield of AO in the binding to α_1-AG (namely more than twenty times) was observed (Fig. 1). We thus found that AO was suitable as a fluor-

escent probe for α_1-AG. On the other hand, AODB bound to both human serum albumin and α_1-AG with similar affinities K_d = approximately 0.25 μM (unpublished observation). Although AODB has a much higher affinity for α_1-AG than AO does, it is thus inferior to AO in terms of specificity.

In the conventional binding measurements using fluorescent dyes, the concentrations of bound and unbound dyes can be calculated by obtaining the fluorescence quantum yield of the bound dye [15]. The fluorescence quantum yield is usually obtained by fluorescence titration, in which a fixed amount of the dye is titrated with increments of the protein until no further increase in fluorescence is observed. However, in the present analysis of the AO binding to α_1-AG, such titration could not be continued until the fluorescence of AO became constant due both to the relatively low affinity of AO and α_1-AG and to the relatively high fluorescence background by α_1-AG itself. Therefore, the $K_{d,app}$ was obtained according to the method of Wang and Edelman [16] only from the titration of a fixed amount of α_1-AG with increments of AO as described in Materials and Methods. In such analysis, however, it is assumed that the concentration of the total AO is close to that of the unbound AO, namely that the bound fraction of AO is small over the whole range of AO concentrations. This assumption was validated by measuring the AO binding to α_1-AG by equilibrium dialysis under the same condition (the concentration of α_1-AG is 4.3 μM and that of AO ranged from 11 to 75 μM). The bound fraction of AO was less than 0.13 at any AO concentration.

The stoichiometry of AO binding to α_1-AG was determined to be 0.79 by equilibrium dialysis, suggesting that α_1-AG has only a single binding site for AO. Recently, Muller and Stillbauer [4] indicated that a single common binding site with high affinity on α_1-AG mediates the binding of various basic drugs such as CPZ, PL and IPM. Therefore, we examined whether the fluorescent dye, AO, and these basic drugs bind to a common or a separate site on α_1-AG. As shown in Figs. 3 and 4, all the basic drugs tested in the present study competitively inhibited the AO binding to α_1-AG. In addition, the values of K_d for various basic drugs, which were calculated by the AO method based on the assumption that these basic drugs competitively inhibit the AO binding to α_1-AG, were comparable to those obtained by other methods and from the literature (Table 2). These findings demonstrated that a single binding site on α_1-AG for AO is identical with the "single basic drug binding site" previously identified [4]. However, the inhibition data showed the deviation from the theoretical curve when the concentrations of inhibitors (PL, QD and CPZ) were high (Fig. 2). The most feasible explanation for the deviations is that the existence of a lower affinity site for these basic drugs might decrease the unbound drug concentrations compared to that which is predicted based on the assumption of simple competition. Consequently, the inhibition curve would deviate upwardly from the theoretical one. In fact, QD binding to α_1-AG measured by equilibrium dialysis exhibited both the high and low affinity binding sites (Table 2), supporting the above mentioned mechanism.

The location of this basic drug binding site on α_1-AG has not been clarified yet. El-Gamel *et al.* [12] suggested that tyrosine and tryptophan residues and a free sulfhydryl group might be involved in the binding of dipyridamole to α_1-AG and that this site is located in a hydrophobic structure of α_1-AG. Our present result, that CPZ quenched the intrinsic fluorescence of α_1-AG which might come from tryptophan and/or tyrosine residues, also supports their suggestion.

We also tried to apply the fluorescent dye, AO, to measure the α_1-AG concentration in human serum. As shown in Fig. 7B, a relatively good correlation was observed between the AO method and the conventional radial immunodiffusion method. The effects of therapeutic concentrations of various drugs on the determination of α_1-AG by the AO method were investigated. The following drugs were used: CPZ (800 ng/ml), IMP (700 ng/ml), DPM (700 ng/ml), QD (10 μg/ml), PL (5 μg/ml), LC (25 μg/ml), DZP (300 ng/ml), PB (80 μg/ml), SA (300 μg/ml), TB (300 μg/ml), WF (3 μg/ml) and progesterone (2 μg/ml). No drugs investigated in the present study disturbed the determination of α_1-AG by the AO method. Neither bilirubin ($\leq$ 3 mg/dl) nor hemoglobin ($\leq$ 2 mg/ml) disturbed the determination. Various basic drugs listed above, which were shown to displace AO from the binding site on α_1-AG (Figs. 2–4), thus did not interfere with the determination of α_1-AG over these concentrations. Two reasons might be considered for this lack of interference. First, the basic drugs in serum were finally diluted by approximately nine times when the fluorescence intensity was measured, and the final concentration of the drugs was much lower than the K_I value. Second, the fluorescence intensity was measured in the presence of an excess amount of AO (90 μM), and therefore the inhibitory effect by basic drugs may be neglected.

The fluorometric method with AO, developed in the present study, has the advantage that only a short time (at most 20 min) is required; therefore, it may be useful when monitoring α_1-AG concentration in serum of individual patients. In addition, it may be used to determine α_1-AG concentration in serum of experimental animals. On the other hand, the conventional radial immunodiffusion requires longer time (at least 1 day) and is not so easily applicable to experimental animals, since the antibodies against α_1-AG of animals are not commercially available. We have not examined whether this method can be used to determine the α_1-AG concentration in the serum of various patients. Future studies thus should be focused on the clarification of the specificity of this method.

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