A Simple and Rapid Fluorometric Determination Method of α_1-Acid Glycoprotein in Serum Using Quinaldine Red

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We examined different fluorescent probes suitable for fluorometric determination of α_1-acid glycoprotein (AGP) in serum. Quinaldine red (QR) was shown to bind strongly and selectively to AGP. Taking advantage of the enhanced fluorescence of QR in the presence of AGP, we developed a direct method for the determination of serum AGP without removal of other serum proteins such as albumin. AGP concentrations in serum of healthy volunteers and patients correlated well with results from the conventional single radial immunodiffusion (SRID) method (r = 0.93, slope = 1). The newly developed method is faster and has a larger analytical concentration range than the SRID method. This method can also be used to determine AGP in serum of experimental animals, and it can serve to monitor AGP serum concentrations for pharmacokinetic evaluation of basic drugs.

KEY WORDS: α_1-acid glycoprotein; fluorescent probe; quinaldine red; single radial immunodiffusion method.

INTRODUCTION

α_1-Acid glycoprotein (AGP), a member of the lipocalin family, serves as a carrier or receptor for small lipophilic compounds (1). It is an important binding protein for basic drugs (2) and hormones (3) in plasma. Although its physiological role is unresolved, it has been suggested that it interacts with the immune system (4). AGP is one of the major positive acute-phase reactants, and its plasma level can vary considerably in various pathological conditions (5). Normal AGP levels range between 40 and 100 mg/100 mL, but AGP values can increase to 300 mg/100 mL with cancer (6) and acute inflammation (7). Won and Baumann (8) showed that human keratinocytes and monocytes regulate the synthesis of AGP in human hepatic cells. The large variations in AGP serum level may cause large variations in the unbound serum levels of basic drugs.

AGP is commonly measured by a single radial immunodiffusion (SRID) method based upon immunoprecipitin. Recently, Sugiyama *et al.* (9) reported an assay of serum AGP with the use of a fluorescent dye, auramine O, after deproteinization with sulfosalicilic acid. However, both methods are time-consuming. We examined fluorescent probes suitable for fluorometric determination of AGP. Quinaldine red, 2-[2-[4-(dimethylamino)phenyl]-l-ethylquinolium iodide (QR; Fig. 1), was found to bind specifically to AGP with a remarkable enhancement of its fluorescence. The enhanced fluorescence of QR bound to AGP was exploited for the direct quantitative determination of serum AGP.

MATERIALS AND METHODS

Materials

Serum samples were obtained from 25 healthy subjects (22 to 27 years old) and 150 patients (25 to 53 years old) who were under a variety of clinical protocols of Kumamoto University Medical School and Shimada Hospital Kumamoto, Japan. Twenty five patients had cancer, 28 had hepatic disease, and 33 had renal disease. A number of medications were used for the patients, but the normal volunteers were not given any medications. Blood samples (10 mL) were drawn by direct vein puncture and kept at room temperature for 1 hr. The samples were thus centrifuged at 1500 *g* for 15 min. The serum fraction was collected and frozen at −40°C until analysis. Human AGP (MW 44,100), human serum albumin (HSA; MW 66,500), γ-globulin (γ-GB; 110,000), 5α-androstane-3,17-dione (Androstanedione), and dansyl-DL-norleucine (DNSL) were obtained from Sigma Chemical Co. (St. Louis, MO). QR, Quinaldine (QD), and quinaldic acid (QA) were purchased from the Tokyo Kasei Co. (Tokyo) and then recrystallized from H_2O-EtOH before use. 2-(4-Dimethylaminostyryl)-*n*-ethylpyridinium iodide (DASP) and 4-(4-diethylstyryl)-*n*-methylpyridinium iodide (4-DASP) were obtained from the Funakoshi Co. (Tokyo). Acridine orange-10-dodecylbromide (AODB), methylene blue (MB), fluorescein (FRC), and sulfosalicylic acid (SSA) were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Warfarin-K (WF) was supplied by Eisai Co (Tsukuba, Japan). Pamaquine (PQ) was purchased from the Nippon Bulk Yakuhin Co. Ltd. (Osaka, Japan). Auramine O (AO) was obtained from the Eastman Kodak Co. (Rochester, NY) and was recrystallized gently from 0.02 M NaCl.

Apparatus

Absorption spectra were recorded on a Shimadzu UV-240 spectrophotometer (Kyoto, Japan). Fluorescence measurements were made on a Jasco FP-770 fluorescence spectrophotometer (Tokyo).

Measurement of Quantum Yield

The quantum yields were assessed according to the method of Imamura *et al.* (10).

Equilibrium Dialysis (ED)

Dialysis experiments were performed using a Sanko plastic dialysis cell (Fukuoka, Japan). The two cell compartments were separated by Visking cellulose membranes. Protein solution was poured into one compartment and the drug solution was poured into the opposite compartment. After

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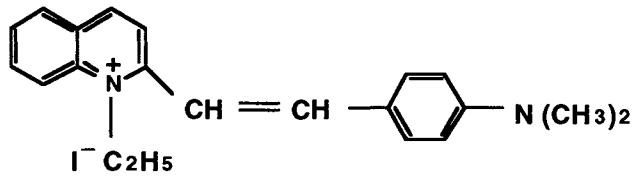

Fig. 1. Structure of quinaldine red.

dialysis at 25°C for 6 hr, QR concentrations in each compartment were assayed spectrophotometrically (wavelength at 496 nm). Bound fraction was corrected for adsorption of drugs on membrane by studying the system without serum or serum protein. Bound drug concentrations and bound fractions were calculated as follows:

bound concentration (D_b) = total drug concentration (before filtration) (D_t) − filtered drug concentration/f_a

bound fraction (B_f) = D_b/D_t

where f_a is the ratio of the concentration after ED without serum or serum protein with respect to the concentration. The f_a of QR was 0.95.

Determination of Binding Parameters

The fluorometric titration method was essentially the same as described previously (10). The results obtained by above methods were analyzed according to the Scatchard equation (11).

Procedures for Determination of AGP Concentration in Serum

QR Method. Stock solutions were prepared with the following substances: (A) 1/15 *M* sodium phosphate buffer, pH 7.4 (standard buffer solution); (B) AGP solution (0–3 mg/mL) in the standard buffer; (C) HSA solution (80 mg/mL) in the standard buffer; (D) γ-GB (34 mg/mL) in the standard buffer; (E) 5α-androstane-3,17-dione (15×10^{-6} *M*) in the standard buffer; and (F) QR solution (1.5×10^{-3} *M*) dissolved in methanol.

For AGP standard curves, appropriate volumes of standard buffer (solution A; 100–0 μL) were adjusted with solution B (an AGP solution; 0–100 μL) to 100 μL (solution G) and then vortexed for 1 min.

Fifty microliters of HSA solution (solution C) and γ-GB (solution D) each were added to solution G (solution H), and solution H was then diluted with solution E (5.8 mL), so that the final concentrations of HSA and γ-GB were 120 times more dilute than the corresponding stock solution. Three milliliters of the diluted solution H was taken into a cuvette for fluorescence measurement as the blank fluorescence intensity (F_1) at 590 nm with the excitation wavelength 496 nm. QR solution (solution C; 100 μL) was then added to the solution with a microsyringe and mixed. Fluorescence intensity (F_2) was again measured at the same excitation and emission wavelength. The difference of F_2 and F_1 gives the fluorescence of AGP alone. Thus a standard curve was constructed by plotting $F_2 - F_1$ versus AGP concentration.

For sample analysis 100 μL of serum was used, and instead of 5.8 mL of solution E, 5.9 mL of standard buffer (solution A) was added.

SRID Method. Five microliters of serum was incubated for 48 hr in commercially prepared plates (Hoechst, Japan) that contained rabbit antibodies. The diameter of the resultant precipitate was measured with an optical micrometer, and the serum concentration of AGP was determined from the standard table.

RESULTS

The fluorescence quantum yield of several probes was measured in the presence of serum protein in pH 7.4 phosphate buffer (Table I). The fluorescence quantum yield of QR was more strongly enhanced upon the addition of AGP than with other probes. In contrast, the fluorescence quantum yield of QR-HSA system and in buffer was negligible (Fig. 2) and lower than that of other probes (Table I). QR binding to AGP and HSA was calculated using the fluorometric titration method. The association constants (K) obtained by the fluorescence method of QR to AGP and HSA were found to be $4.2 \times 10^5\ M^{-1}$ ($n = 1.1$) and $1.6 \times 10^3\ M^{-1}$ ($n = 1.8$) for AGP and HSA, respectively (10). However, binding to γ-GB could not be obtained because of low fluorescence yields.

In addition, we obtained the binding percentage of QR to AGP, HSA, and γ-GB using ED. The results are shown in Table II. The binding percentages of QR for AGP, HSA, and

Table I. Fluorescence Characteristics of Interaction Between Serum Proteins and Probes[a]

	Probe												
Protein	QR	DNSL	PQ	FRC	AODB	WF	MB	AO	PR	QA	QD	DASP	4-DASP
AGP													
Φ[b]	0.699	0.094	—[c]	—	0.518	0.116	—	0.096	—	—	—	0.563	0.151
λ_{em} (nm)	587	388			518	388		502				565	570
HSA													
Φ[b]	0.006	1.173	—	—	0.740	0.106	—	0.083	—	—	—	0.070	0.042
λ_{em} (nm)	594	503			519	393		500				570	580
Φ_{AGP}/Φ_{HSA}	116.5	0.1			0.7	1.09		1.16				8.0	3.6

[a] [AGP] = [HSA] = 1.0×10^{-6} *M*. [Probe] = 1.0×10^{-6} *M*.
[b] Fluorescence quantum yield.
[c] No enhancement.

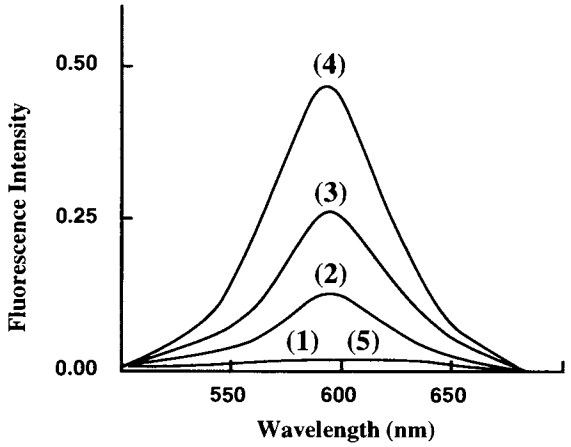

Fig. 2. Fluorescence spectra of QR in the presence of serum protein. (1) [AGP] = 0; (2) [AGP] = 0.5×10^{-6} *M*; (3) [AGP] = 1.0×10^{-6} *M*; (4) [AGP] = 2.0×10^{-6} *M*; (5) [HSA] = 2.0×10^{-6} *M* and [QR] = 2.0×10^{-6} *M*.

γ-GB were 23, 3.4, and 1.2%, respectively. QR specifically and strongly bound to AGP. The standard curve (AGP–QR system) owing to determination of AGP concentration in serum is shown in Fig. 3. A good relationship was observed between isolated AGP concentration and fluorescence intensity ($r = 0.99$). However, the AGP concentration in serum as determined by the AGP-QR method was higher than that obtained by the SRID method. Therefore we searched for endogenous substrates such as steroids (for example, testosterone, etiocholane-17β-ol-3-one, epiandrosterone, progesterone, and 4-androsterone-3,17-dione) and fatty acids (for example, oleic acid, palmitic acid, and linolenic acid) which could increase the fluorescence intensity of QR bound to AGP. 5α-Androstane-3,17-dione (androstanedione) was found to be responsible for the enhanced fluorescence intensity of QR bound to AGP. Androstanedione per se did not fluoresce in the buffer, AGP, and QR solutions. The enhancement of fluorescence intensity was dependent on the amount of androstanedione added in the AGP-QR mixture. However, the fluorescence intensity reached a maximum value above 15 μ*M* androstanedione (Fig. 4). Therefore, if androstanedione above 15 μ*M* is used, the fluorescence intensity of QR should depend on the AGP concentration alone, yielding a new standard curve for AGP–QR–androstanedione (Fig. 3). However, the fluorescence intensity values were lower than those in serum (Fig. 3). Moreover, we observed enhanced fluorescence intensity of the AGP–QR–androstanedione system upon the addition of HSA and γ-GB. As the fluorescence intensity of QR to AGP in the AGP–QR–androstanedione–HSA–γ-GB system was greater than that in the AGP–QR–androstanedione system, a new standard curve with AGP–QR–androstanedione–HSA–γ-GB was constructed (Fig. 3). In this case, the values

Table II. Binding of QR (%) to Serum Protein

AGP (2 mg/100 mL)	HSA (80 mg/100 mL)	γ-GB (17 mg/100 mL)
23.0	3.4	1.2

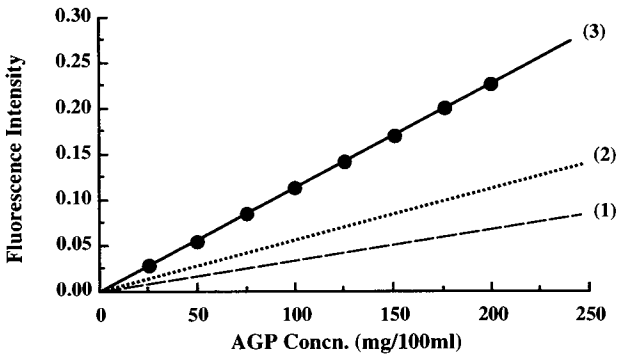

Fig. 3. Standard curves for measurement of AGP. (1) Observed standard curve by QR method (AGP–QR system). (2) Observed standard curve by QR method (AGP–QR–androstandione system). (3) ——, Theoretical standard curve by SRID method; ●, observed standard curve by QR method (AGP–HSA–γ-GB–androstanedione–QR system).

of fluorescence intensity agreed with the fluorescence intensity of QR in serum (Fig. 3). We use the term "standard artificial serum" for the AGP–QR–androstanedione–HSA–γ-GB system. We thus determined the AGP concentration in serum using QR with standard artificial serum and the SRID method. The effects of androstanedione on the binding of QR for AGP are summarized in Table III. The binding percentage of QR to AGP was reduced by the addition of androstanedione to the AGP–QR system. However, the binding of QR was recovered by adding HSA and γ-GB into the AGP–QR–androstanedione system, as shown by the increase in fluorescence.

Because the serum concentration of albumin (40 mg/mL) is much higher than that of AGP (0.5 to 1 mg/mL), we examined whether there was any effect of the fluorescence which might arise from the HSA–QR complex. The removal of major proteins (HSA and γ-GB) from serum was done according to the method of Routledge *et al.* (12). Treatment of human serum with SSA removed almost all the proteins except for AGP. This was confirmed as follows. First, in sodium dodecyl sulfate (SDS)-gel electrophoresis, the SSA-

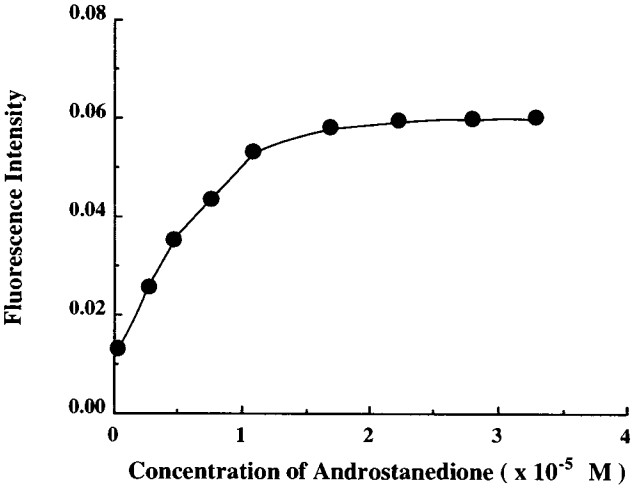

Fig. 4. Effects of androstanedione on the fluorescence intensity of the AGP–QR system. [AGP] = 1.0×10^{-6} *M*; [QR] = 2.5×10^{-6} *M*.

Table III. Effect of 5-Androstane-3-17-dione (Androstanedione) of Serum Protein on Binding Percentage for QR–AGP Systems[a]

	AGP–QR	AGP–QR–Androstanedione	AGP–QR–Androstanedione–HSA–γ-GB
Binding (%)	23.0	5.0	24.0

[a] [AGP] = 4.5×10^{-6} *M*; [QR] = 6.0×10^{-5} *M*; [HSA] = 1.2×10^{-5} *M*; [androstanedione] = 1.3×10^{-6} *M*; [γ-GB] = 1.1×10^{-4} *M*.

treated human serum showed a single band corresponding to commercially available purified AGP. Then the fluorescence intensity of an excess amount of QR (25 μ*M*) was measured using both SSA-treated and untreated protein samples. The fluorescence intensity of QR in HSA or γ-GB was negligible after treatment with SSA, while in AGP there was a small change in fluorescence before and after treatment with SSA. The principle of the assay for AGP established in the present study is as follows. Most of the serum proteins except for AGP were removed by treatment with SSA, followed by centrifugation. Subsequently, AGP left in the supernatant fraction was determined by two direct methods (QR method and SRID method) and the fluorescence probe method (using AGP-QR system standard curve) after treatment with SSA. The result is shown in Fig. 5. We obtained a good relationship between the two direct methods (Fig. 5A) and the fluorescence probe method (Fig. 5B) after treatment with SSA. These data indicated that the newly established QR method determined only the AGP concentration in serum (slope = 1, r = 0.99).

To confirm the utility of the QR method, we measured AGP concentrations in serum of 175 volunteers (healthy volunteers, 25 samples; disease patients, 150 samples) by both QR and SRID methods. As shown in Fig. 6, a good correlation was observed between the two methods (slope = 1, r = 0.93). A comparison between the QR and the SRID methods is shown in Table IV.

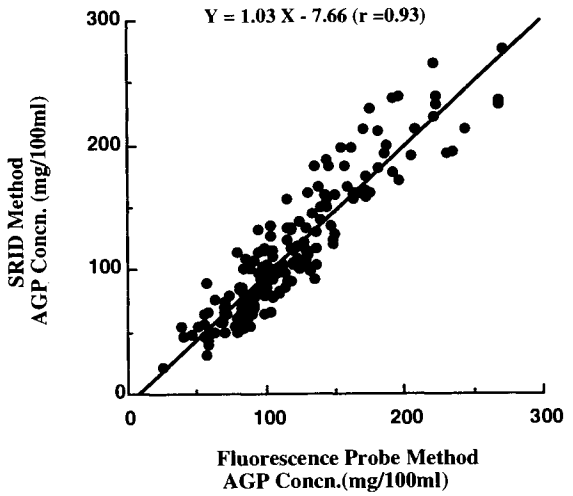

Fig. 6. Relationship of the AGP concentrations determined by the SRID method and by the fluorescence probe method.

DISCUSSION

The fluorescence intensity of QR was enhanced as a function of the AGP concentration at a longer wavelength (Fig. 2). Moreover, QR did not have fluorescence in the buffer containing HSA solution (Fig. 2). The binding parameters and binding percentage of QR to serum protein were calculated not only for AGP but also for HSA to determine whether the fluorescence enhancement of QR in the presence of AGP was actually due to the binding to AGP. Because the serum concentration of HSA (4.0 g/100 mL) is much higher than that of AGP, the fluorescence which arose from the HSA–QR complex cannot be neglected. QR specifically and strongly bound to AGP compared with other proteins in serum, which suggests that QR is superior to other probes. The apparent AGP concentration in serum as determined from the standard curve (AGP–QR system) was higher than that determined by the SRID method. This phe-

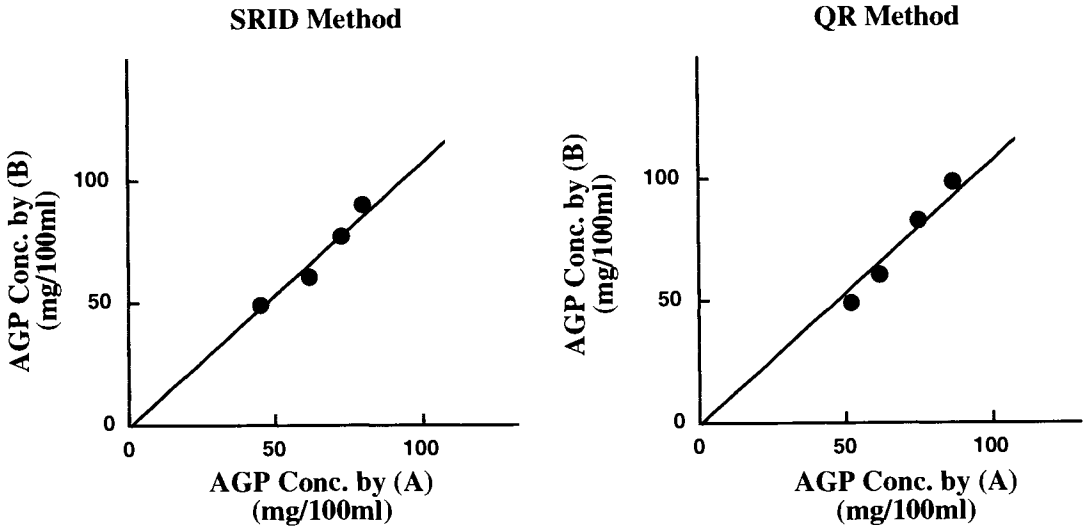

Fig. 5. Relationship of the AGP concentrations determined by two direct methods (A) and the fluorescence probe method (B) after treatment with sulfosalicylic acid.

Table IV. Comparison of New Method and SRID Method

	New method	SRID method
Measuring time	Short (1 hr)	Long (50 hr)
Operation procedure	Simple	Complex
Detection range	5–500 mg/100 mL	13–199 mg/100 mL
Operation cost	Inexpensive	Expensive
CV[a]	<3%	<10%

[a] Coefficient of variation.

nomenon suggests that some endogenous components in serum affect the fluorescence character of AGP. However, only androstanedione enhanced further the fluorescence intensity of QR bound to AGP. However, the fluorescence intensity of the AGP–QR–androstanedione system did not agree with that obtained with QR in serum, while the fluorescence intensity obtained by adding HSA and γ-GB to the AGP–QR–androstanedione system agreed well with that obtained with QR in serum. The effects of the precursor of this steroid hormone, metabolite, and other components in serum on the fluorescence emission of AGP–QR system were insignificant.

While we considered the mechanism by which androstanedione increased the fluorescence intensity of QR on AGP, we examined the effect of androstanedione on the binding of QR for AGP (Table III). The binding percentage of QR to AGP was reduced by adding androstanedione to the AGP–QR system. However, interestingly, the binding of QR was recovered by adding HSA and γ-GB to the AGP–QR–androstanedione system. These data indicate that the binding of QR to isolated AGP was as strong as that of QR to artificial serum; moreover, QR bound mainly to AGP in standard artificial serum. It was assumed that the enhancement of fluorescence intensity of QR in standard artificial serum was caused by the increase in binding of QR to AGP.

Recently, an increased level of AGP with abnormal carbohydrate chains has been reported to be present in the serum of cancer patients (13,14). However, the influences of AGP modification by carcinoma, sex difference, and age can be neglected since a good correlation was observed between the QR method and the SRID method (Fig. 5). We must consider the interference of basic drugs in the determination of the AGP concentration in serum of patients of various diseases. However, to minimize potential interference we diluted the basic drugs in serum approximately 60 times and performed fluorescence measurement experiments in the presence of an excess amount of QR (25 μ*M*). The interference thus can easily be overcome using this new method. In addition, this newly developed method can be used to determine the AGP concentrations in serum of experimental animals. On the other hand, the SRID method is not easily applicable to experimental animals, since the antibodies against AGP of animals are not commercially available. Moreover, the SRID method is time-consuming (24 to 48 hr) and suitable only for a narrow concentration range of AGP. Therefore, this newly developed method can be useful when monitoring the AGP concentration in serum of different kinds of patients for evaluation of pharmacokinetic properties of some basic drugs and animal experiments.

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