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Fluorescence quenching of serum albumin by rifamycin antibiotics and their analytical application

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ABSTRACT: In neutral medium, rifamycin antibiotics such as rifapentin (RFPT), rifampicin (RFP), rifandin (RFD) and rifamycin SV (RFSV) can bind with human serum albumin (HSA) and bovine serum albumin (BSA) to form complexes, resulting in the quenching of the intrinsic fluorescence ($\lambda_{ex}/\lambda_{em}$ = 285/355 nm) of the BSA and HSA. The quenching intensity (ΔF) is directly proportional to the concentration of the rifamycin antibiotics. Therefore, a new analytical method was established to determine trace rifamycin antibiotics. The method had fairly high sensitivity and the detecting limits (3σ) for RFPT, RFP, RFD and RFSV were 0.85, 0.98, 1.83, 1.89 ng/mL, respectively, for the HSA system and 0.76, 0.89, 1.55, 1.77 ng/mL, respectively, for the BSA system. All relative standard deviations (RSDs) were <3.8%. In this work, the characteristics of the fluorescence spectra were studied and the optimum reaction conditions and influencing factors were investigated. The influence of coexisting substances was tested and the results showed that the method had good selectivity and could be applied to determine trace rifamycin antibiotics in medicine capsules and urine samples. Taking the RFSV–serum albumin system as an example, the reaction mechanisms, such as binding constants, binding sites, binding distance and the type of fluorescence quenching, were investigated. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: rifamycin antibiotics; serum albumin; fluorescence quenching; analytical application

INTRODUCTION

Rifamycin SV (RFSV), rifandin (RFD), rifampin (RFP) and rifapentine (RFPT) are common rifamycin antibiotics whose molecular structures are shown in Fig. 1. Rifamycin has antibacterial activity to Gram-positive bacteria, Gram-negative bacteria, anaerobe and leprosy bacillus and is often used to treat tuberculosis. Its antibacterial mechanism is that it can restrain the reproductive function of RNA, controlled by DNA, and can prevent the synthesis of thallus mRNA (1). After rifamycin medicines enter the human body, they first bind with serum albumin and then reach the receptor site through storage and transportation via the blood. Serum albumin is the medicine carrier in the plasma and has important physiological, physical and chemical functions. Much information about the transfer, distribution and metabolization of a medicine *in vivo* can be obtained by studying the interaction of the medicine and serum albumin *in vitro*. Related studies can afford advantageous conditions for understanding the functional mechanism, toxicity and metabolizability of medicines and establish better foundations for developing new analytical methods for proteins and medicines. These studies have therefore received much attention in such fields as life sciences, pharmacology and clinical

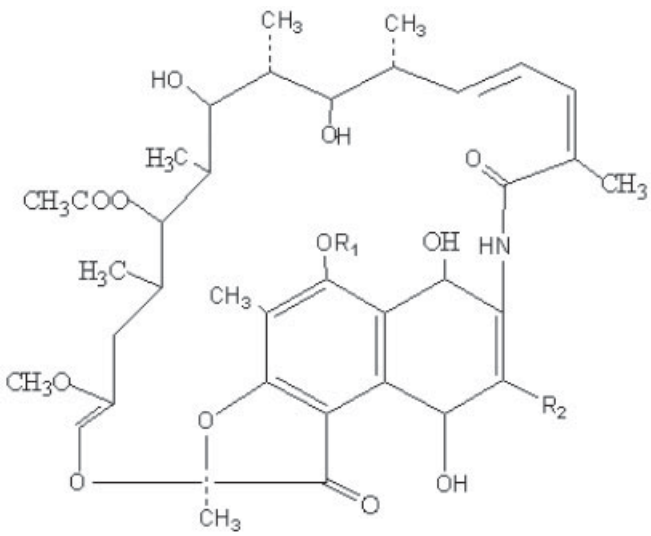

Figure 1. Molecular structure of rifamycins. Rifamycin SV, R_1 = Na, R_2 = H; Rifampin, R_1 = H, R_2 = CH=N–N N–CH_3; Rifapentine, R_1 = H, R_2 = CH=N–N N–; Rifandin, R_1 = H, R_2 = N N–CH2–

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medicine, etc. (2–4). At present, methods such as spectrophotometry (5, 6), microorganism valency (7), balance dialysis (8, 9), chemiluminescence (10), electrochemistry (11, 12), hig-pressure liquid chromatography (HPLC) (13–17) and nuclear magnetic imaging (NMR) (18, 19) have been applied to the study of the interaction of rifamycin antibiotics with proteins. A fluorescence method has been used to investigate the fluorescence quenching, fluorescence photosensitivity, energy transfer and the effect of experimental conditions on medicines and proteins (18). The fluorescence quenching of serum albumin by rifamycin antibiotics and its analytical application has not yet been reported.

In this study we found that, under neutral or weak basic conditions, the reaction of rifamycin antibiotics with human serum albumin (HSA) and bovine serum albumin (BSA) could result in fluorescence quenching, and the quenching intensity (ΔF) was directly proportional to the drug concentration. The method had fairly high sensitivity and the detection limits (3σ) for RFPT, RFP, RFD and RFSV were 0.85, 0.98, 1.83 and 1.89 ng/mL, respectively, for the HSA system and 0.76, 0.89, 1.55 and 1.77 ng/mL, respectively, for the BSA system. All the RSDs were <3.8%. Therefore, a new fluorescence quenching method for the determination of trace amounts of rifamycin antibiotics, using HSA or BSA as probes, could be developed. It was more sensitive than other methods, e.g. the detection limits were as follows: for spectrophotometry, 0.4 μg/mL (6); for microorganism valency, 4.2 μg/mL (7); for chemiluminescence and electrochemistry, 3.2 ng/mL (10) and 5.0 ng/mL (12); and for HPLC, 0.16 μg/mL, 1.9 μg/mL, 4.9 μg/mL, 0.2 μg/mL, 3.0 μg/mL (13–17). This method was applied successfully to determine rifamycin antibiotics in drugs and human urine samples. In addition, taking the RFSV–serum albumin system as an example, the fluorescence quenching reaction mechanisms, such as binding constant, binding sites, binding distance, type of fluorescence quenching and reasons for fluorescence quenching were investigated.

EXPERIMENTAL

Regents and apparatus

The concentrations of BSA (99%; Sino-American Biotechnology Company) and HSA (20%; Wuhan Alpha Biotechnologies Co. Ltd) stock standard aqueous solutions were 5.0×10^{-4} mol/L. 1.5×10^{-4} mol/L RFSV (99.9%; National Pharmaceutical and Biological Products Control Institute) stock solution was prepared with water, and those of RFP, RFD and RFPT (99.8%; Hebei Xingang Pharmaceutical Co. Ltd) were first dissolved in a little chloroform and methanol, then 5.0×10^{-5} mol/L working solutions were prepared by diluting the stock solution with water. Britton–Robinson (BR) buffer solution was prepared by mixing the mixed acid (composed of 0.04 mol/L H_3PO_4, HAc and H_3BO_3) with 0.2 mol/L NaOH according to a certain proportion. All reagents were analytical grade and double-distilled water was used throughout.

An F-4500 fluorescence spectrophotometer (Hitachi, Japan) was used for recording and measuring the fluorescence spectra. An UV-3010 ultraviolet-visibility near-infrared spectrophotometer (Hitachi, Japan) was used for recording the absorption spectra. A PHS-25C pH meter (Shanghai Yulong Analytical Instrument Plant) was used for adjusting pH values.

General process

Into a 10 mL volumetric flask were added 1.0 mL 5.0×10^{-5} mol/L BSA or HSA solution, 2.0 mL BR buffer solution, pH 7.6, and certain amounts of rifamycin antibiotics solution. The resulting solution was diluted with double-distilled water to the mark and mixed thoroughly. After 20 min at 30°C, the fluorescence and excitation spectra were recorded using a F-4500 fluorescence spectrophotometer 10 nm excitation and emission slit widths. At the maximum emission wavelength (λ_{em}), the fluorescence intensity of the binding product (F) and that of the reagent blank as the protein solution without antibiotics (F_0) were measured ($\Delta F = F_0 - F$).

RESULTS AND DISCUSSION

Fluorescence spectra

Under the experimental conditions, BSA and HSA both have fluorescence and their maximum excitation (λ_{ex}) and emission (λ_{em}) wavelengths are at 285 and 355 nm, respectively. The fluorescence intensity of BSA is stronger than that of HSA. Rifamycin antibiotics themselves have no fluorescence, but when they react with HSA or BSA to form complexes, the fluorescence of HSA and BSA was quenched. It can be seen from Fig. 2 that the fluorescence quenching effects of the four rifamycin antibiotics differ, in the order RFPT > RFP > RFD > RFSV. In the HSA system, RFSV and RFD could not result in a change of the fluorescence spectra of HSA, but RFP and RFPT caused a red-shift of 15 nm compared with the maximum emission wavelength of HSA (Fig. 2A). In the BSA system, except for RFSV, other three rifamycins caused red-shifts of 10 nm compared with the maximum emission wavelength of BSA (Fig. 2B). It was discovered that the fluorescence quenching intensity (ΔF) of the BSA (or HSA) system had a linear relationship with the concentration of the rifamycin antibiotics. Therefore, the fluorescence quenching method can be used to determine the drugs.

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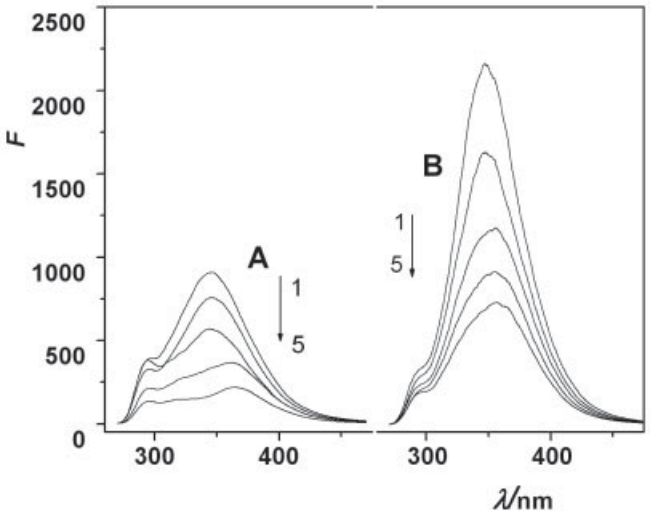

Figure 2. Fluorescence spectra. (A) Rifamycins–HSA system, pH 7.6; 1, HSA (1.0×10^{-5} mol/L); 2, RFSV–HSA; 3, RFD–HSA; 4, RFP–HSA; 5, RFPT–HSA. (B) Rifamycins–BSA system, pH 7.6; 1, BSA (1.0×10^{-5} mol/L); 2, RFSV–BSA; 3, RFD–BSA; 4, RFP–BSA; 5, RFPT–BSA. In both systems, the concentration of all rifamycins was 3.0×10^{-6} mol/L.

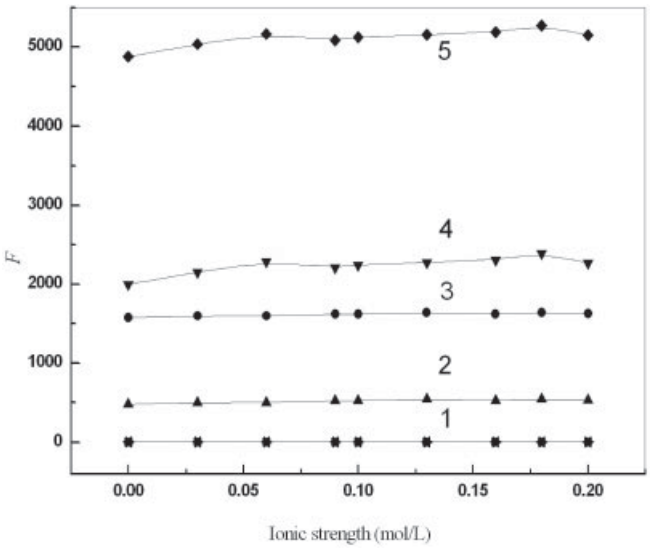

Figure 4. Effect of ionic strength on fluorescence intensity (F). Concentrations: 1, RSFV (5.0×10^{-6} mol/L); 2, HSA (5.0×10^{-5} mol/L) + RFSV; 3, HSA; 4, BSA (5.0×10^{-5} mol/L) + RFSV; 5, BSA.

Optimum reaction conditions

Taking the RFSV–BSA (or –HSA) system as an example, the optimum conditions of the fluorescence quenching were tested.

Effect of acidity. The effects of acidity on the fluorescence intensity of the BSA, HSA, BSA–RFSV and HSA–RFSV systems are shown in Fig. 3, from which it can be seen that BSA and HSA themselves have the strongest fluorescence at pH 7.0–10.0. When they combine with RFSV, the fluorescence is strongly quenched. In this work, pH 7.6 BR buffer solution was selected, and the optimum amount of buffer solution was found to be 1.0 mL.

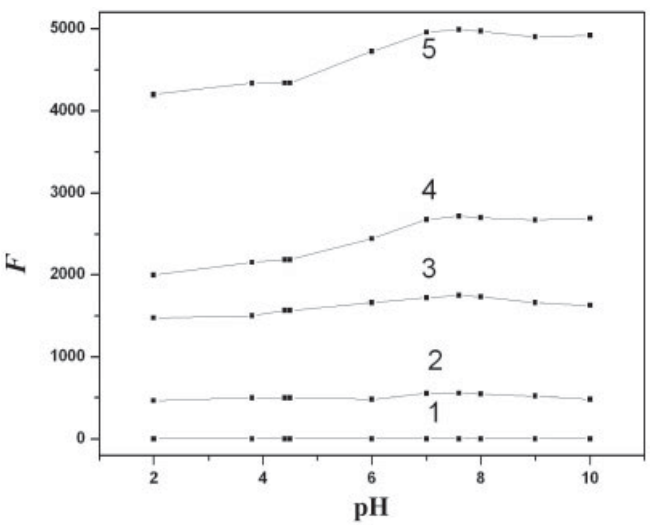

Figure 3. Effect of pH on fluorescence intensity (F). Concentrations: 1, RFSV (5.0×10^{-6} mol/L); 2, RFSV + HSA (5.0×10^{-5} mol/L); 3, RFSV + BSA (5.0×10^{5} mol/L); 4, HSA; 5, BSA. $\lambda_{ex}/\lambda_{em}$ = 285/355 nm.

Effect of ionic strength. Ionic strength was adjusted by using NaCl solution. It was found that 0–0.2 mol/L NaCl solution had little effect on the fluorescence intensity of BSA, HSA, BSA–RFSV and HSA–RFSV solutions (Fig. 4). It shows the reaction can occur under loose conditions, e.g. the reaction was independent of the ionic strength. At the same time it also offers useful information on the interactive forces between RFSV and HSA or BSA, namely, under neutral conditions, negatively charged RFSV reacted with the same charged BSA or HSA mainly by virtue of the hydrogen bonding, van der Waals' interaction and a hydrophobic effect, not through electrostatic attraction, because the latter was much influenced by ionic strength.

Effect of protein concentration. The effects of the concentrations of BSA (or HSA) on fluorescence quenching intensity (ΔF) were investigated. It can be seen from Fig. 5 that when the concentration of BSA (or HSA) was low, ΔF was weak. As the protein concentration increased, ΔF gradually increased. When the concentration of BSA (or HSA) reached 5.0×10^{-5} mol/L, ΔF was maximal, but if the protein concentration was $>5.0 \times 10^{-5}$ mol/L the fluorescence intensity would decrease because of the self-quenching of the fluorescence by the protein. The optimum concentration of BSA or HSA was therefore 5.0×10^{-5} mol/L.

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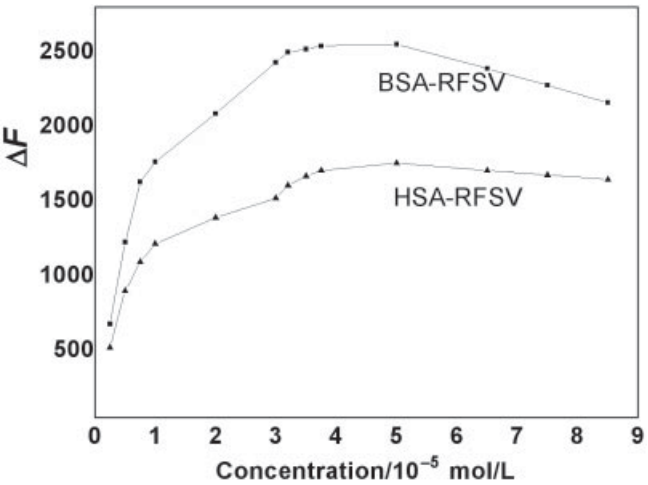

Figure 5. Influence of concentration of BSA and HSA on fluorescence quenching. Concentrations: HSA ($0.1–9 \times 10^{-5}$ mol/L); BSA ($0.1–9 \times 10^{-5}$ mol/L); RFSV (5.0×10^{-6} mol/L).

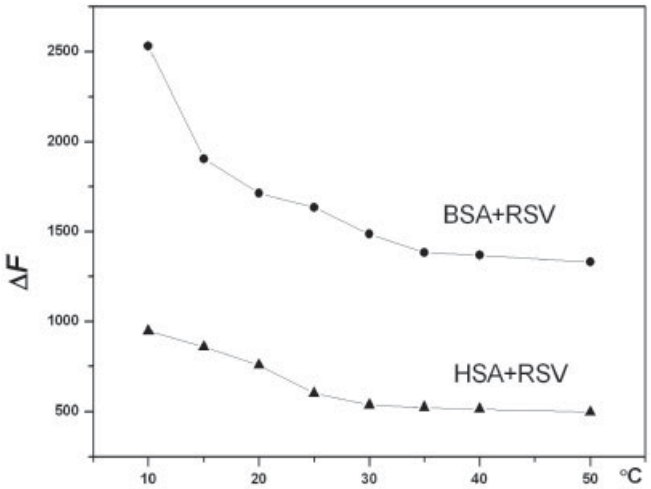

Figure 6. Influence of temperature on fluorescence quenching of HSA–RFSV and BSA–RFSV systems.

Effect of temperature. The effects of temperature on ΔF of the BSA–RFSV and HSA–RFSV systems were examined in the range 10–50°C (Fig. 6). For the BSA system, there was a turning point at 20°C. This showed that the fluorescence would quench more strongly at low temperatures. Over 20°C, with an increase of temperature, the fluorescence would quench more slowly. The HSA–RFSV system showed the same phenomenon, but its ΔF was smaller than that of the BSA system. Considering the small difference of the relative fluorescence quenching intensities, in the range 30–40°C, we selected the optimum temperature range as 30–40°C.

3.3 Sensitivity and selectivity of the method

Relation between the fluorescence quenching intensity and the concentration of rifamycins. Under the optimum reaction conditions, ΔF was directly proportional to the concentration of rifamycin antibiotics. Therefore, a new analytical method was established for the determination of rifamycin antibiotics. The fluorescence quenching values ($\Delta F = F_0 - F$) at 355 nm were measured by setting the excitation wavelength at 285 nm. Then ΔF was plotted vs. the corresponding concentration of the medicines. The linear regression equation, correlation coefficients, linear ranges and detection limits are listed in Table 1, which shows that the detection limits (3σ) are <1.89 ng/mL and RSD ($n = 11$) <3.8% for 1 μg/mL drugs. The method has higher sensitivity than that reported for HPLC [detection limit (3σ) of 4.6–5.4 ng/mL (15)].

Effects of coexisting substances. Taking the RFSV–HSA system as an example, the effects of various foreign substances, such as amino acids, glucide, uric acid and some metal ions, on the determination of RFSV were investigated when the concentration of RFSV was 1.0 μg/mL. The tolerable amount of foreign species was found when the relative error was within ±5%. The results are shown in Table 2, from which it can be seen that certain amounts of monosaccharide, oligosaccharide, polysaccharides such as starch, amino acids and common metal ions did not interfere with the determination. Therefore, the method had good selectivity.

Table 1. Some parameters for the calibration graphs and detection limits for rifamycin antibiotics

System	Regression equation (μg/mL)	Linear range (μg/mL)	Detection limit/ 3σ (μg/mL)	Correlation coefficient	RSD* (%)
BSA–RFPT	$\Delta F = 879.71C + 1.92$	0.008–50.0	0.00076	0.9996	2.8
BSA–RFP	$\Delta F = 802.76C + 2.51$	0.009–50.0	0.00089	0.9991	3.7
BSA–RFD	$\Delta F = 738.51C + 3.13$	0.016–40.0	0.00155	0.9998	3.0
BSA–RFSV	$\Delta F = 688.46C + 4.34$	0.018–40.0	0.00177	0.9995	3.8
HSA–RFPT	$\Delta F = 589.89C + 1.38$	0.010–40.0	0.00085	0.9997	2.7
HSA–RFP	$\Delta F = 532.13C + 2.14$	0.011–40.0	0.00098	0.9990	3.5
HSA–RFD	$\Delta F = 486.32C + 3.34$	0.019–35.0	0.00183	0.9996	2.9
HSA–RFSV	$\Delta F = 433.88C + 4.52$	0.019–35.0	0.00189	0.9994	3.2

*Concentration (C) of the drug is 1.0 μg/mL ($n = 11$).

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Table 2. Effect of coexisting substances (RFSV, 1.0 μg/mL)

Substance	C (μg/mL)	Substance	C (μg/mL)
Na^+	800	Maltose	200
K^+	900	Gluocose	200
Co^{2+}	12	Urea	300
Cu^{2+}	10	Uric acid	300
Mg^{2+}	250	Glycine	50
Al^{3+}	16	L-Phenylalanine	20
Fe^{3+}	10	L-Histidine	30
Ni^{2+}	15	L-Tryptophan	40
Ca^{2+}	200	Cellulase	10
Zn^{2+}	40	Fructose	300
Pb^{2+}	20	Starch	100
NH_4^+	100	Vitamin C	100

C, concentration.

Analytical application

Determination of the concentration of rifamycin antibiotics in capsules. The shells of 10 capsules of rifampin (Chongqing Tiansheng Medicine Pharmacy Ltd), rifandin (Chengdu Jinhua Medicine Pharmacy Ltd) and rifapentin (Leshang Sanjiuchangzheng Medicine Pharmacy Ltd), labelled 150 mg/capsule, were removed and the powder contents of the 10 capsules were mixed. 20.0 mg mixing powder was weighed precisely and dissolved in the mixing solution of chloroform and methanol, then transferred into a 50.0 mL volumetric flask and diluted with double-distilled water to the mark. This solution was then diluted 250 times. 2.0 mL of the diluted solution was taken and determined according to the experimental method. The detected result was consistent with that of spectrophotometry (5) (see Table 3). It can be seen that this method has high accuracy and can be applied to the analysis of medicines.

Determination of rifampin in urine samples. Clinical study indicates that about 15% original rifomycin drugs and 6–15% deacetyl metabolite were excreted from urine, and ≥90% of the drugs can bind with albumen. Urine samples were obtained from the first urination of two patients 3 h after taking 600 mg rifampin. The proteins in the urine samples were first removed, then the drug concentrations were determined and the recoveries tested. Table 4 shows that the method had good recoveries and the residue of rifampins in urine samples was minimal.

Fluorescence quenching mechanism

Types of fluorescence quenching and relative parameters. Taking the RFSV–HSA and RFSV–BSA systems as examples, the fluorescence quenching mechanism caused by the interaction of rifamycin antibiotics with proteins was investigated. There are two types of fluorescence quenching, dynamic and static (20). Dynamic quenching should satisfy the Stern–Volmer equation (21):

$$F_0/F = 1 + K_q\tau_0\,[Q] = 1 + K_{SV}\,[Q] \quad (1)$$

where F_0 is the unquenching fluorescence, F is the fluorescence intensity at $[Q]$, K_q is the bimolecular quenching constant, K_{SV} is the Stern–Volmer quenching constant, τ_0 is the fluorescence lifetime in the absence of quencher [the τ_0 of the biological macromolecule is 10^{-8} s (22)], and $[Q]$ is the concentration of quencher.

The static quenching should satisfy the Lineweaver–Burk relationship (22):

Table 3. Results for the determination of content in rifamycin antibiotic capsules

		This method		Spectrophotometry*	
Sample (capsule)	Labelled (mg/capsule)	Found (n = 5) (mg/capsule)	RSD (%) (n = 5)	Found (n = 5) (mg/capsule)	RSD (%) (n = 5)
RFD	150	148.4	3.9	146.9	2.8
RFPT	150	163.5	2.1	159.5	2.5
RFP	150	157.5	2.9	149.7	2.6

*Reference (5).

Table 4. Results of the determination of rifampin in urine samples

Method	Sample No.	Found (μg/mL)	Added (μg/mL)	Total amount (μg/mL)	Recovery (%)	RSD (%) (n = 5)
Fluorescence quenching	1	0.5 ± 0.4	1.00	1.59 ± 0.6	106.0	3.8
	2	ND**	1.00	1.03 ± 0.4	103.0	3.9
Spectrophotometry*	1	0.45 ± 0.3	1.00	1.42 ± 0.4	98.0	2.8
	2	ND**	1.00	0.95 ± 0.3	95.0	3.2

*Reference (5).
**ND, not detected.

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Table 5. Linear equations for different quenching types

System	Quenching type	Temperature (°C)	Stern–Volmer linear equation	Correlation coefficient
BSA–RFSV	Dynamic state	30	$F_0/F = 0.7966 + 1.069 \times 10^5\,[Q]$	0.9853
		40	$F_0/F = 1.091 + 8.524 \times 10^4\,[Q]$	0.9845
	Static state	30	$1/(F_0 - F) = 0.0004608 + 2.5701 \times 10^{-10}\,(1/[Q])$	0.9992
		40	$1/(F_0 - F) = 0.0005373 + 4.7474 \times 10^{-9}\,(1/[Q])$	0.9984
HSA–RFSV	Dynamic state	30	$F_0/F = 0.9043 + 7.095 \times 10^4\,[Q]$	0.9832
		40	$F_0/F = 0.9092 + 9.772 \times 10^4\,[Q]$	0.9820
	Static state	30	$1/(F_0 - F) = 0.001348 + 4.5074 \times 10^{-9}\,(1/[Q])$	0.9994
		40	$1/(F_0 - F) = 0.001525 + 3.7371 \times 10^{9}\,(1/[Q])$	0.9981

$$\frac{1}{(F_0 - F)} = \frac{1}{F_0} + \frac{1}{K_{LB}F_0[Q]} \quad (2)$$

where K_{LB} is the static quenching binding constant of the interaction of medicine molecule with the protein biological macromolecule.

The relationships of F_0/F with $[Q]$ and $1/(F_0 - F)$ with $1/[Q]$ for the RFSV–BSA and RFSV–HSA systems were measured at different temperatures. Table 5 shows the linear equations at different temperatures.

K_q and K_{SV} can be calculated from equation (1) (Table 5). It is well known that the maximum diffusing controlled collision quenching constant of various quenchers to the biological macromolecule is $K_q' = 2.0 \times 10^{10}$ L/mol/s (22). Obviously, in Table 5, the rate constant of the fluorescence quenching of RFSV to BSA and HSA was bigger than the K_q' controlled by diffusion. Therefore, the quenching was not initiated by dynamic collision.

At the same time, K_{LB} of the two systems was calculated according to equation (2) at different temperatures (Table 5). The correlation coefficients of static quenching calculated according to equation (2) were all better than those of dynamic quenching calculated according to equation (1), i.e. the relationship of $1/(F_0 - F)$ with $1/[Q]$ for the two systems had better linearity than the relationship of F_0/F with $[Q]$, so the quenching of the two systems is of the static type. On the other hand, K_{LB} decreased as temperature increased, which also showed that the quenching was static, initiated by the production of new material. It can be seen from the section on Effect of temperature on fluorescence quenching, above, that fluorescence quenching decreased as temperature increased, which also verified that the reaction was of the static quenching type.

Binding constant and binding sites. For static quenching, the relationship of the fluorescence intensity and the concentration of quencher can be expressed as follows:

$$\lg\frac{F_0 - F}{F} = \lg K_0 + n\lg[Q] \quad (3)$$

where F_0 and F are the fluorescence intensities before and after the quencher was added, K_0 is the binding constant of the quenching reaction, and n is the number of binding sites. The relationship of $\lg(F_0 - F)/F$ with $\lg[Q]$ for the RFSV–BSA and RFSV–HSA systems were measured separately at different temperatures and linear equations obtained. For the RFSV–BSA system, $n = 1.27$ (30°C) and 0.81 (40°C); for the RFSV–HSA system, $n = 1.14$ (30°C) and 1.03 (40°C). The binding constants (K_0) of the two systems were the same as K_{LB} calculated according to equation (2).

Molecular interaction distance of RFSV with BSA and HSA. Figure 7 shows the overlapped fluorescence spectra of BSA or HSA and the absorption spectrum of RFSV. The overlap of the fluorescence spectrum of the donor and the absorption spectrum of the acceptor shows that energy transfer exists here. According to Förster's non-radiative energy transfer theory, the distances between binding site and the fluorescence emission group in the protein molecule can be determined (23–25). The efficiency (E) of energy transfer is related not only to the

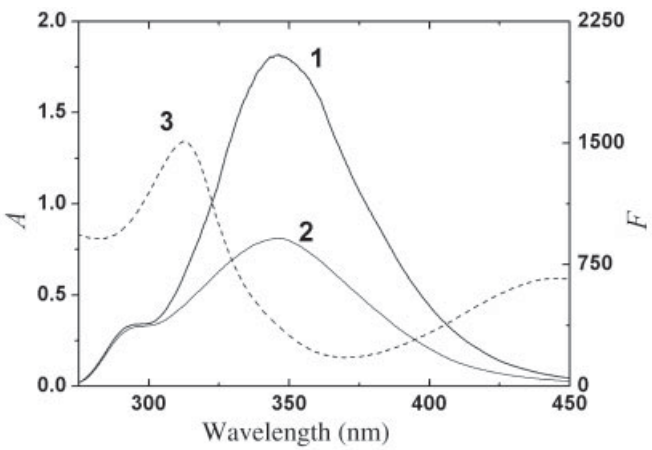

Figure 7. Overlap of the fluorescence spectrum of serum albumin and the absorption spectrum (A) of a drug; 1, fluorescence spectrum of BSA; 2, fluoresence spectrum of HAS; 3, absorption spectrum of RFSV.

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distance (r) between the donor and the acceptor, but also to the critical energy transfer distance (R_0):

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r^6) \quad (4)$$

where F is the fluoresence intensity of the binding product when the donor and the acceptor are mixed at the same concentration and F_0 is the fluorescence intensity of the acceptor without the donor. E can be calculated from the experimental value of F and F_0.

R_0 is the critical distance when the transfer efficiency E is 50%:

$$R_0^6 = 8.8 \times 10^{25}\ K^2 N^{-4}\ \Phi\ J \quad (5)$$

where K^2 is the spatial orientation gene of the dipole [2/3 (24)], N is the refractive index of the medium (1.336), Φ is the fluorescence quantum yield of the donor, whose value is 0.118, J is the overlapped integral of the fluorescence emission spectra of the donor (protein) and the absorption spectrum of the acceptor (drug) when the concentration of the donor and acceptor are the same. Therefore:

$$J = \Sigma F(\lambda)\varepsilon(\lambda)\ \lambda^4 \Delta\lambda / \Sigma F(\lambda)\ \Delta\lambda \quad (6)$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ and $\varepsilon(\lambda)$ is the molar absorptivity. The integral of the HSA system ($J = 3.456 \times 10^{-17}$ cm^3•L/mol) and the BSA system ($J = 6.098 \times 10^{-17}$ cm^3•L/mol) was calculated by integrating the overlapped spectra. R_0 could be calcuated according to equation (5), then r could be calculated according to $E = R_0^6/(R_0^6 + r^6)$. The energy transfer efficiency (E) and the binding distance (r) were calculated as 31.22% and 2.92 nm for the BSA system, and 40.28% and 1.11 nm for the HSA system.

Reasons for fluorescence quenching. The fluorescence of the protein was caused by some amino acid residues in the peptide chain of the proteins. Among these amino acids residues, tryptophan contributes mostly to the fluorescence, and its maximum excitation (λ_{ex}) and emission (λ_{em}) wavelengths are located at 285 nm and about 350 nm. Tyrosine also contributes to the fluorescence, and its maximum excitation and emission wavelengths are at 275 and 303 nm, respectively, while its relative fluorescence intensity is 1% that of tryptophan (26). Phenylalanine has very weak fluorescence and makes little contribution to the fluorescence of proteins. Because the maximum excitation and emission wavelengths of BSA and HSA are at 285 and 355 nm, respectively, the fluorescence of BSA and HSA is caused mainly by the tryptophan residues (27). The fluorescence of BSA and HSA will obviously be quenched when RFSV binds with protein, which is the result of the formation of the static complex between RFSV and the tryptophan residue in the peptide chain of the protein. The number of binding sites of RFSV with HSA or BSA is one, i.e. a molecule of RFSV binds with a tryptophan residue in the peptide chain. In neutral or weak basic medium, RFSV exists as a univalent ion, and it has strong hydrophobicity for its alkyl chain and aryl group, and the tryptophan residue in the peptide chain of the protein belongs to the nonpolar R group, therefore it binds with RFSV mainly by virtue of hydrogen bonding, the hydrophobic effect and van der Waals' interactions. This is in accordance with our conclusion. The binding mode could be as follows: the oxygen atom in the phenolic group of RFSV forms a hydrogen bond with the tertiary ammonium group (–NH) of the indole ring in tryptophan, and the hydroxyl group in the alkyl chain of RFSV forms a hydrogen bond with the carbonyl group in the tryptophan residue. These hydrogen bonds and the hydrophobic effect make the binding effect of RFSV with HSA or BSA very strong (K can reach an order of magnitude of 5–6 at 30°C). It can be presumed that the binding effect of RFSV and the tryptophan residue results in the fluorescence quenching of protein. We calculated the charge density of each atom in the binding system of RFSV with the tryptophan residue in the peptide chain, using the HF/3-21G of *ab initio* quantum chemistry calculations (Fig. 8), and the results show that the hydrogen atom of the hydroxyl group in the alkyl chain of RFSV has a larger positive charge density, and it easily forms a hydrogen bond with the carbonyl group in the tryptophan residue, which has larger negative charge density. At the same time, the negative oxygen ion in the naphthalene ring of RFSV forms a hydrogen bond with the ammonium group (–NH) of the indole ring in the tryptophan residue. The calculation results are also in accordance with our conclusion.

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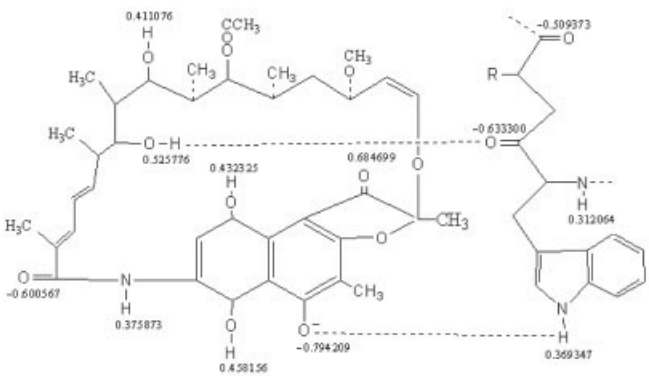

Figure 8. Binding diagram of rifamycin SV and tryptophan residue.

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