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Full Paper

Effects of the Interaction of Rifamycin SV with Serum Albumins on the Resonance Rayleigh Scattering Spectra and Their Analytical Application

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In pH 4.5—4.8 Britton-Robinson buffer solution, rifamycin SV (*i.e.* rifamycin sodium) can react with serum albumin such as human serum albumin (HSA) and bovine serum albumin (BSA) to form macromolecular complexes by electrostatic attraction and hydrophobic force. As a result, the resonance Rayleigh scattering (RRS) of the drug was enhanced remarkably and the RRS peaks were at 374 and 552 nm. The enhancement of RRS (ΔI) is directly proportional to the concentration of HSA or BSA. The linear ranges and the detection limits are 0.03—6.0 μg/mL and 9.0 ng/mL for HSA, and 0.01—8.0 μg/mL and 2.0 ng/mL for BSA, respectively. In this work, a sensitive, selective, simple and fast method for the determination of trace amounts of serum albumin by RRS technique has been developed, which was applied to the determination of serum albumin in the synthesized samples and human urine samples with satisfactory results.

Keywords resonance rayleigh scattering (RRS); rifamycin SV (RFSV); serum albumin; protein determination

Introduction

Rifamycin SV (RFSV) is a broad-spectrum antibiotic of rifamycins and is often used to treat tuberculosis. The antibacterial mechanism is that it can restrain the reproductive function of RNA polymerase controlled by DNA and prevent the synthesis of the thalli mRNA. It has antibacterial activity to many kinds of pathogenic microorganisms.[1] After they enter the human body, the rifamycin medicines bind with serum albumin and then reach the receptor site through storage and transportation of the blood that produces pharmacological action. Therefore, it is great significant to study the interaction of medicine with serum albumin *in vitro* for understanding the physiological action and physical and chemical function of serum albumin, and for knowing the functionary mechanism, toxicity and metabolizability of medicines *in vivo* at the molecular level. The serum albumin is the medicine carriers in the plasma and it has the important physiological function and physical and chemical function. It can afford some important information about the transfer, distribution and metabolization of the medicine *in vivo*. At present, there are such methods for the study of interaction of RFSV with proteins as spectrophotometry,[2-4] balance dialysis method,[5,6] chemilumenescence,[7] electrochemical analysis,[8,9] HPLC[10-12], NMR[13,14], *etc*. The interaction research of RFSV with serum albumin by RRS has not been reported.

Resonance Rayleigh scattering (RRS) is a newly developed analytical technique, which has characteristics of both scattering and electron absorption spectra. It is related to the forced vibration of the molecular electron in the electromagnetism field of the incident light, and also related to the transition of electron. The RRS originates from the Rayleigh scattering and the absorption spectra, while it is different from them and forms a new characteristic spectrum. Therefore, it is different from the fluorescence and the absorption spectra, which can offer much more spectral characteristics of the binding mode, action mode, reaction characteristic, *etc*. of the medicines with biological macromolecules[15] than RRS can. In recent years, the RRS method has been successfully used to determine biomacromolecules.[16-19] Because RRS spectral technology is more sensitive than other methods in the non-covalent bond interaction such as association, aggregation, assembly, dipole-dipole, static and hydrophobic interaction, which is extremely advantageous in the research of determination and characterization of biomacromolecule, and the mechanism of reaction, it has been widely used in the determination and characterization of biomacromolecules. In this work, we found that, in weak acidic media, the RRS of RFSV, HSA and BSA alone was very weak, but after

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they bound with each other, the RRS was strongly enhanced. The enhanced RRS intensity ΔI is directly proportional to the concentration of BSA or HSA in a certain range. Based on this, a new RRS method was established to determine BSA and HSA and the sensitivity is higher than those of the other methods.

Experimental

Regeants

The concentrations of BSA (Sino-American Biotechnology Company，99%) and HSA (Wuhan Alpha Biotechnologies Co., Lid., 20%) stock standard solutions were 5.0×10^{-4} and 1.5×10^{-4} mol/L, respectively. And the concentration of RFSV (Tri-Lion Pharmaceutical Co., Lid. Harbin) stock solution was 5.0×10^{-4} mol/L. The working solutions were prepared by diluting the stock solution with water. The Britton-Robinson (B-R) 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 at a certain proportion. All reagents were of analytical grade. Doubly distilled water was used throughout.

Apparatus

An F-4500 fluorescence spectrophotometer (Hitachi, Japan) was used for recording and measuring the RRS spectra and the intensities. A UV-3010 VU-Vis 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 value.

Experimental procedure

Into a 10 mL volumetric flask were added 2.0 mL of pH 4.8 BR buffer solution, certain amounts of BSA (or HSA, pH 4.5) solution and appropriate amounts of RFSV solution. The resulting solution was diluted with doubly distilled water to the mark and then was inverted for 20 times to ensure complete mixing. At last, the RRS spectra of above solution were recorded with synchronous scanning at $\lambda_{ex}=\lambda_{em}$ by the F-4500 fluorescence spectrophotometer. The absorption spectra were obtained by the UV-3010 spectrophotometer using the buffer solution as the reference solution.

Results and discussion

RRS spectra of RFSV and serum albumin

The RRS spectra of the reaction systems of RFSV with HSA or BSA are shown in Figures 1. It can be seen from Figure 1 that the RRS intensity of RFSV, HSA or BSA alone was quite weak. When the RFSV bound with HSA or BSA, the RRS was enhanced and the enhanced intensity (ΔI) was directly proportional to the concentration of BSA or HSA in a certain range. It can be applied in the determination of BSA or HSA. The RRS peaks of the two systems are both located at 374 and 552 nm. However, the linear relation of ΔI with the concentration of HSA or BSA at 374 nm is better than that at 552 nm.

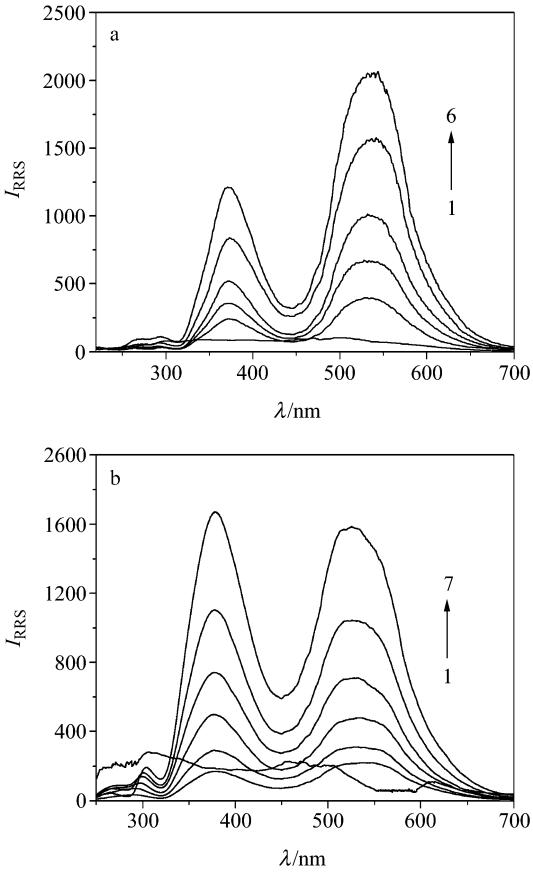

Figure 1 (a) RRS spectra of RFSV-BSA. pH 4.8. (1) BSA (0.3 μg/mL); (2) RFSV (5.0×10^{-5} mol/L); 3—6, RFSV-BAS (μg/mL): 3, 0.5; 4, 1.0; 5, 1.5; 6, 2.0. (b) RRS spectra of RFSV-HAS. pH 4.5. (1) HSA (0.3 μg/mL); (2) RFSV (5.0×10^{-5} mol/L); 3—7, RFSV-HSA (μg/mL): 3, 0.3; 4, 0.6; 5, 0.9; 6, 1.5; 7, 2.5.

Optimum reaction conditions

Effect of the acidity: The effect of the acidity on the RRS intensity (I_{RRS}) of the reaction system is shown in Figure 2. It can be seen from Figure 2 that, in pH 3.0—10.0, the RRS intensities of RFSV, HSA or BSA alone change little, and in pH 2.0—3.0, they enhance slightly probably owing to their self-aggregation. However, the acidity has strong influence on the RRS intensity of the binding products such as RFSV-HSA and RFSV-BSA. The optimum pH ranges for the two systems are 3.5—4.8 and 4.0—5.2, respectively. Because there are more strong acidic side groups or weak basic side groups near the two neighboring sulfur bridges in BSA structure than those in HSA, it is more advantageous to form the non-covalent bond nearby its isoelectric point. In this work, 1.5 mL of pH 4.5 and 4.8 BR buffer solutions were chosen for RFSV-HSA and RFSV-BSA systems.

Effect of the ionic strength: Effect of ionic strength controlled by NaCl solution on the RRS intensity was tested. When the concentration of NaCl was between

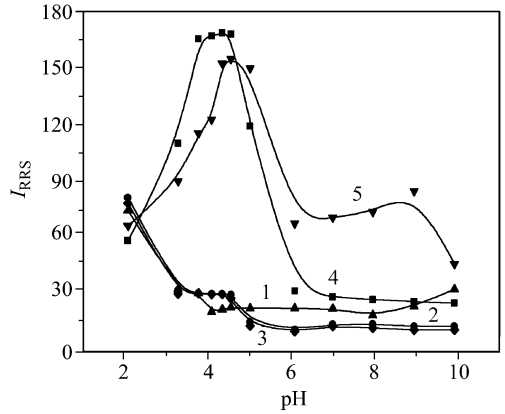

Figure 2 Influence of acidity on RRS intensity. (1) RFSV (5.0×10^{-5} mol/L); (2) HSA; (3) BSA; (4) RFSV-HSA; (5) RFSV-BSA. All concentrations of serum albumare are 2.0 μg/mL.

0.0 and 0.2 mol/L, the RRS intensity of RFSV, HSA or BSA alone was little influenced, and those of RFSV-BSA and RFSV-BSA systems were affected to different extent. The ionic strength has strong effect on the RRS intensity of RFSV-HSA system, which decreased quickly with the increase of ionic strength. However, the RRS intensity of the RFSV-BSA system had little change when the concentration of NaCl was lower than 0.15 mol/L. Because the pH 4.8 of RFSV-BSA system is closer to the isoelectric point of the protein, and the hydrophobic interaction is bigger than the electrostatic attraction, the effect of ionic strength on RFSV-BSA system is less. Therefore, inorganic salt should be avoided for RFSV-HSA system.

Effect of the concentration of RFSV: We investigated the effect of the concentration of RFSV on the RRS intensity by controlling the concentration of HSA and BSA solution at 3.0×10^{-5} mol/L. The optimum concentration ranges of RFSV were 3.5×10^{-5}—8.5×10^{-5} mol/L for HSA-RFSV system and 4.0×10^{-5}—8.5×10^{-5} mol/L for BSA-RFSV system. We selected 5.0×10^{-5} mol/L RFSV solution for both systems.

Stability of RRS intensity (ΔI): At room temperature, the reaction could complete within 25 min and the enhanced RRS intensity reached the maximum, which can keep stable for 36 h. Therefore, we should determine BSA or HSA in 25 min.

Interaction of RFSV with the serum albumin and the effect on RRS

The binding ratio of RFSV to the serum albumin was determined by using a molar ratio method and the result showed that RFSV : HSA＝1.33, RFSV : BSA＝1.17 (molar binding ratio).

In addition, the interaction of RFSV with protein was discussed through the Scatchard analysis.

The Scatchard formula can be expressed as:[20]

$$\frac{r}{c_f} = nK - rK$$

where, r is the molar binding ratio of RFSV to the serum albumin, c_f is the concentration of the uncombined RFSV, n is the maximum binding number, K is the binding constant of RFSV with the serum albumin. It can be shown from Figure 3 that the linear equations of RFSV-BSA and RFSV-HSA systems are $r/c_f=1.17\times10^7-8.99\times10^6 r$ ($R=0.9968$, $n=5$) and $r/c_f=2.64\times10^6-2.18\times10^6 r$ ($R=0.9959$, $n=5$), respectively. Therefore, for RFSV-BSA and RFSV-HSA systems, the binding number site of the two systems are 1.30 and 1.21, and the binding constants are 8.99×10^6 and 2.18×10^6 L/mol, respectively.

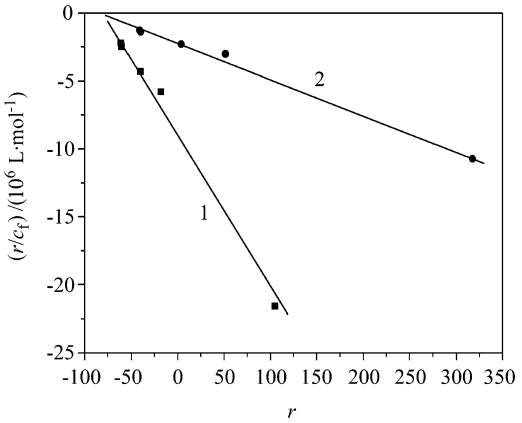

Figure 3 Scatchard chart of RSV-BSA and RSV-HSA system. (1) RFSV-BSA (pH 4.8); (2) RFSV-HSA (pH 4.5).

The results determined by above two methods are the same, *i.e.*, the binding ratio of RFSV to the serum albumin (HSA and BSA) is close to 1∶1.

Because the optimum pH range is below the isoelectric point of HSA or BSA, the residue of amino acids in the peptide is positively charged. And under this condition, the RFSV exists as a univalent anion, therefore, proteins and RFSV can bind with each other through electrostatic attraction, besides, the hydrogen bond, van der waals forces and the hydrophobic interaction also make important contribution.

The interaction of RFSV with the serum albumin can enhance the intensity of RRS, and the reasons for the RRS enhancement are as follows:

(i) Resonance scattering enhancement effect: The absorption spectra of RFSV, HSA, BSA, RFSV-HSA and RFSV-BSA solutions are shown in Figure 4. HSA and BSA have weak absorption at 280 nm. RFSV has strong absorptions at 450 and 320 nm and a valley at 280 nm. When HSA or BSA was added into RFSV solution, the absorbances at 450 and 320 nm increased, and an enhanced peak at 280 nm appeared. And the absorption coefficient $\varepsilon(\lambda)$ increased. Because I_{RRS} is equal to $KCMI_0$ and K is equal to kf $[\varepsilon(\lambda)]$, ΔI_{RRS} is enhanced as the $\varepsilon(\lambda)$ increased.[21]

Taking the RFSV-HSA system as a real sample, the absorption spectra and the RRS spectra are shown in Figure 5. The strong absorption peaks were at 450 and 320 nm and the RRS intensities at 374 and 552 nm were

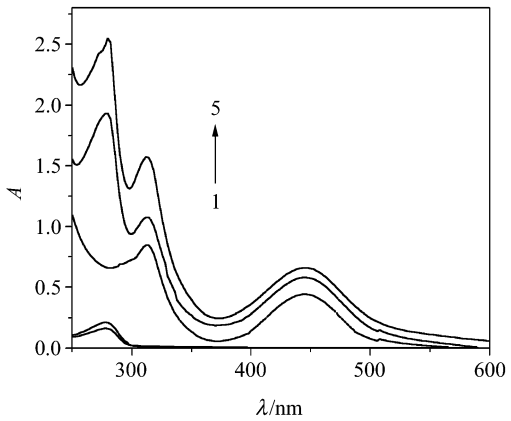

Figure 4 Absorption spectra of RSV-HSA and RSV-BSA reaction system. (1) HSA (3.0 μg/mL); (2) BSA (3.0 μg/mL); (3) RFSV (5.0×10^{-5} mol/L); (4) RFSV-HSA; (5) RFSV-BSA.

enhanced, which shows that the RRS spectrum is located in its absorption band. The inverted reflection relation of the peak and the valley in the two spectra shows that the RRS is a special spectral phenomenon resulting from the reabsorption and rescattering of the Rayleigh scattering. When the molecular absorption frequency is equal to scattering frequency, the resonance and energy transfer will happen, which leads to the enhancement of RRS.[22]

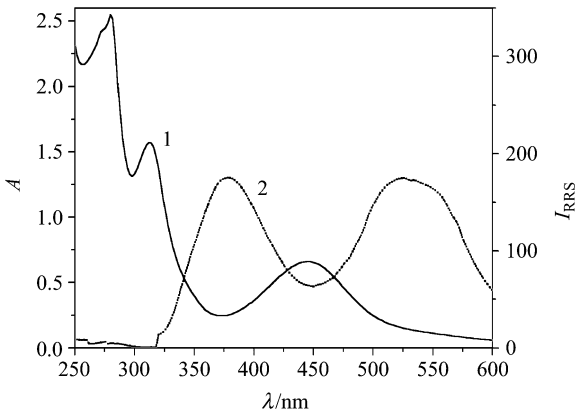

Figure 5 Comparison of absorption spectra (1) and RRS spectra (2) of RFSV-HSA system. pH 4.5; RFSV (5.0×10^{-5} mol/L)-HSA (3.0 μg/mL).

(ii) Surface enhanced scattering effect: When the pH is below the isoelectric point of the serum albumin, there is electrostatic interaction between the positively-charged BSA or HSA and the negatively-charged RFSV to some extent. RFSV is a large circle organic compound containing naphthalene moiety and has strong hydrophobicity. Proteins have many hydrophobic amino acid residues, and RFSV binds with the amino acid residues mainly through hydrophobic interaction. There will be a hydrophobic interface in the aqueous solution that enhances RRS.[21] This can be verified by the enhancement of RRS of the RFSV-BSA system (Figure 1a). The selected pH values of RFSV-HSA and RFSV-BSA systems are 4.5 and 4.8 and the latter is closer to the isoelectric point of the protein (4.9), and the hydrophobic interaction is bigger than the electrostatic attraction. Therefore, the RRS of the RFSV-BSA system is stronger.[23,24]

Analytical application

Relation between the RRS intensity and the concentration of serum albumin: Under the optimum experimental conditions, ΔI_{RRS} has linear relation with the concentration of the serum albumin. The linear regression equations, correlation coefficients, linear ranges and detection limits are listed in Table 1. Based on those, a new method can be established for the determination of trace serum albumin.

Selectivity: Taking the RFSV-BSA system as a real sample, the effects of foreign substances on the reaction were tested. The results are shown in Table 2. It can be seen that certain amounts of amino acids, starch, urea, ascorbic acid, and glucide have little interference and only some metal ions, such as Fe^{3+}, Al^{3+}, Cu^{2+} have bigger interference.

Analysis of synthesized samples: Taking the RFSV-BSA system as a real sample, four synthesized samples were determined and the results are shown in Table 3, indicating that the method has high accuracy and reproducibility.

Determination of albumin in urine samples: The content of albumin in urine of natural human is about 0—50 μg/mL, which is difficult to determine by common methods. This RRS method can determine the trace albumin in urine conveniently, which provides a simple method for clinical analysis.

Fresh urine samples were collected from the healthy volunteers. These samples were filtrated firstly, and 5.0 mL of filtrate were diluted to 50.0 mL. 2.0 mL of the dilute solution were pipetted into a 10 mL volumetric flask, and this solution was used as a sample solution. According to the experimental method, 5.0 mL 5×10^{-4} mol/mL RFSV solution and pH 4.5 buffer solution were added in order and diluted with water to the mark. ΔI was determined with the method. The recovery was tested using a standard addition method and the results are shown in Table 4. The reason for the low recovery is existence of the negative interference organic substances (such as some carbohydrate, *etc*. in Table 2) in the actual urine samples which have not been removed.

Table 1 Analytical parameters for the determination of different proteins

System (RSV: 5×10^{-5} mol•L^{-1})	Linear equation	Correlation coefficient R (n=6)	Linear range/ (μg•mL^{-1})	Detection limit (3σ)/(ng•mL^{-1})	RSD/% (n=5)
RFSV-BSA	$\Delta I=2.956\times10^{6}c+24.69$	0.9976	0.01—8.0	2.0	3.1
RFSV-HSA	$\Delta I=3.753\times10^{6}c+14.90$	0.9983	0.03—6.0	9.0	3.6

Table 2 Effect of foreign substance

No.	Substance	Concentration	ΔI_{RRS}/%	No.	Substance	Concentration	ΔI_{RRS}/%
1	Na^+	4000	−4.68	13	Maltose	300	−5.44
2	K^+	3500	−5.53	14	Glucose	300	−5.51
3	Co^{2+}	10	−4.71	15	Urea	400	+5.38
4	Cu^{2+}	5.0	−6.75	16	Uric acid	350	+5.72
5	Mg^{2+}	220	+4.89	17	Glycine	50	+5.53
6	Al^{3+}	9.0	+5.34	18	*L*-Phenylalanine	20	+5.22
7	Fe^{3+}	3.1	−5.98	19	*L*-Phistidine	30	+5.27
8	Ni^{2+}	15	−3.96	20	*L*-Tryptophane	40	+4.43
9	Ca^{2+}	200	+4.52	21	Cellulase	10	+5.63
10	Zn^{2+}	40	+3.09	22	Fructose	400	−2.57
11	Pb^{2+}	20	−2.98	23	Starch	80	−3.63
12	NH_4^+	60	+5.10	24	Vitamin C	100	+5.65

[a] The concentration of coexisting foreign substance: μg/mL; c_{BSA}: 2.0 μg/mL ,c_{RFSV}: 5.0×10^{-5} mol/ L, pH 4.8.

Table 3 Analytical results for synthetic samples

Synthesized sample/(μg•mL^{-1})	Coexisting substance/(10^{-6} mol•L^{-1})	Found value/(μg•mL^{-1}) (n=5)	Recovery/% (n=5)	RSD/% (n=5)
BSA 1.0	K^+ 200, Co^{2+} 2.0, glucose 200, glycin 20	1.05	105	1.9
BSA 2.0	Fe^{3+} 2.0, *L*-tryptophan 40, urea 200, Cu^{2+} 2.0	2.04	102	2.2
HSA 1.0	*L*-histidine 20, maltose 200, Al^{3+} 5.0, K^+ 200	0.98	98.0	2.1
HSA 2.0	Mg^{2+} 50, fructose 200, uric acid 200, starch 50	1.99	99.5	2.4

Table 4 Results of determination of trace amounts of HSA in urine samples

No.	Found/(μg•mL^{-1}) (n=5)	Added/(μg•mL^{-1})	Found total amount/(μg•mL^{-1}) (n=5)	Recovery/%	RSD/% (n=5)
Sample 1	0.20±0.15	1.00	1.09±0.6	90.8	3.5
Sample 2	0.10±0.17	1.00	0.98±0.4	89.0	3.2

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