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Complexation of HSA with different forms of antimony (Sb): An application of fluorescence spectroscopy

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ABSTRACT

Antimony (Sb) pollution has been of a great environmental concern in some areas in China. Sb enters human body via drinking water, inhalation and food chain, unavoidably interacts with human serum albumin (HSA) in blood plasma, and consequently does harm to human health. The harmful effects of Sb on human health depend on the Sb species and their binding ability to HSA. In the present study, binding of three forms of Sb with HSA was investigated by excitation-emission matrix (EEM) spectroscopy. All of antimony potassium tartrate, antimony trichloride and potassium pyroantimonate quenched fluorescence of HSA. Values of conditional stability constant K_a ($\times 10^5$/M) for Sb and HSA systems were 8.13–9.12 for antimony potassium tartrate, 2.51–4.27 for antimony trichloride and 3.63–9.77 for potassium pyroantimonate. The binding constant K_b ($\times 10^4$/M) values of HSA with antimony potassium tartrate, antimony trichloride and potassium pyroantimonate were 0.02–0.07, 3.55–5.01, and 0.07–1.08, respectively. There was one independent class of binding site for antimony trichloride towards HSA. There was more than one Sb binding site and negative cooperativity between multiple binding sites for potassium pyroantimonate and antimony potassium tartrate towards HSA. The binding ability of HSA to complex Sb followed the order: antimony trichloride > potassium pyroantimonate > antimony potassium tartrate.

1. Introduction

Antimony (Sb) has been considered as an emerging pollutant recently. Sb is toxic to microorganisms, plants and human being. Sb is widely used to produce medicine, alloys, pigments, polyethylene terephthalate, brake linings, cable covering, ammunitions, bearings and flame retardants [1]. Large amount of Sb has been released into the atmosphere, surface water, underground water and soil environment during the extensive use of Sb in these industries and led to serious Sb pollution [1–4]. Sb has become one global emerging contaminant and this caused more and more concern from the government and scientists [1,5].

Sb is toxic to photosynthetic microbes, plants, animals and human being [6–9]. When Sb enters the human body from polluted air, water or food, it may provoke various toxicological effects. Short-term exposure to Sb can lead to acute disease for gastrointestinal tract, and long-term exposure to Sb may damage blood and liver. Inhalation of Sb can affect the respiratory tract and cardiovascular system, lead to pneumoconiosis and restrictive airway disorders [10].

The serum albumin is the sole carrier of fatty acids and the chief osmoregulator. In the homologous albumins such as human serum albumin (HSA), bovine serum albumin (BSA), pig serum albumin (PSA) and rat serum albumin (RSA), HSA is the most sable one [11]. HSA is the most abundant protein in blood plasma, which constitutes about 60% of the total plasma protein and provides about 80% of the blood osmotic pressure [12]. The typical property of HSA is its ability to bind and transport many endogenous and exogenous substances, such as fatty acids, metabolites, drugs, dyes, pesticides and metal ions [13–24]. Also, it is widely accepted that the degree of affinity between ligand and HSA can dominate its distribution into target tissue, affect its elimination from the body, and finally influence its therapeutic or toxic effects, biotransformation and biodistribution of ligands [25]. Besides, it is proved that the conformation of protein changes by its interaction of ligands, and these changes seem to affect the secondary and tertiary structure of protein [25]. It has been proved that the distribution and free concentration of various ligands can be drastically altered as a result of their binding to HSA.

Sb exists in environment in many kinds of forms and the toxicity of Sb depends on its form. The toxicity of elementary Sb is stronger than its compounds and inorganic Sb has stronger toxicity than organic Sb. The toxicity of Sb(III) compounds is of

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10 times more than Sb(V) compounds [1]. Most studies on toxic effects of Sb focused on drug toxicology due to the wide use of Sb-containing drugs. It was observed that exposure to trivalent forms of Sb led to liver damage, hemolysis, hematuria, apoptosis in human fibroblasts, sister chromatid exchanges and circulatory disease [26]. However, the mechanism of human organ or blood interacting with Sb is still not well known.

Fluorescence spectroscopy has been widely used to study the ligand–protein interaction due to its inimitable sensitivity, rapidity, and simplicity [27]. The objective of this study is to quantitatively study interaction of HSA with different forms of Sb using excitation-emission matrix (EEM) fluorescence spectrometry. Conditional stability constants and binding constants for Sb-HSA system were reported and the possible binding mechanisms were also discussed.

2. Materials and methods

2.1. Materials

HSA (fatty acid free $<0.05\%$) was obtained from Source leaf biological technology company (Shanghai, China), and used without further purification. All other chemicals were of analytical grade. Milli-Q ultrapure water was applied throughout the experiments. Phosphate buffer solution (PBS, 0.1 M) containing NaCl (0.15 M) was used to keep the pH of the solution at 7.4 and the ionic strength at 0.15. Dilutions of the HSA stock solution (1×10^{-4} M) in PBS were prepared immediately before use and stored at 4 °C. Stock Sb solutions (1 mM) were prepared with Milli-Q ultrapure water and antimony potassium tartrate ($(K(SbO)C_4H_4O_6\cdot 1/2\ H_2O$: Sb(III)), antimony trichloride ($SbCl_3$: Sb(III)) and potassium pyroantimonate ($K_2H_2Sb_2O_7\cdot 4\ H_2O$: Sb(V)).

2.2. Methods

All EEM spectra were measured by a fluorescence spectrophotometer (F-7000, Hitach, Japan) equipped with 1.0 cm quartz cell and a thermostat bath. EEM spectra were collected by scanning emission wavelength from 200 to 500 nm at 2 nm increments by varying the excitation wavelength from 200 to 350 nm at 5 nm increments. The width of the excitation/emission slit was set to 5.0 nm and the scanning speed was set to 1200 nm/min. The fluorometer's response to a Milli-Q water blank solution was subtracted from the fluorescence spectra recorded for EPS samples under the same conditions. EEM data were processed using the software Sigmaplot 10.0 (Systat, US).

HSA solutions were titrated with incremental μL addition of 1 mM Sb solution at 308 K. After each addition of Sb solution, HSA solution was fully mixed using a magnetic stirrer for 15 min and the fluorescence spectra were recorded. The equilibrium time was set as 15 min since fluorescence intensities at peaks varied little after 15 min reaction. All the titration experiments were triplicated and the mean values were used.

3. Results and discussion

3.1. Effects of different forms of Sb on fluorescence of HSA

The three-dimensional fluorescence spectra of HSA and HSA-Sb system are shown in Fig. 1. Two fluorescence peaks were obtained in

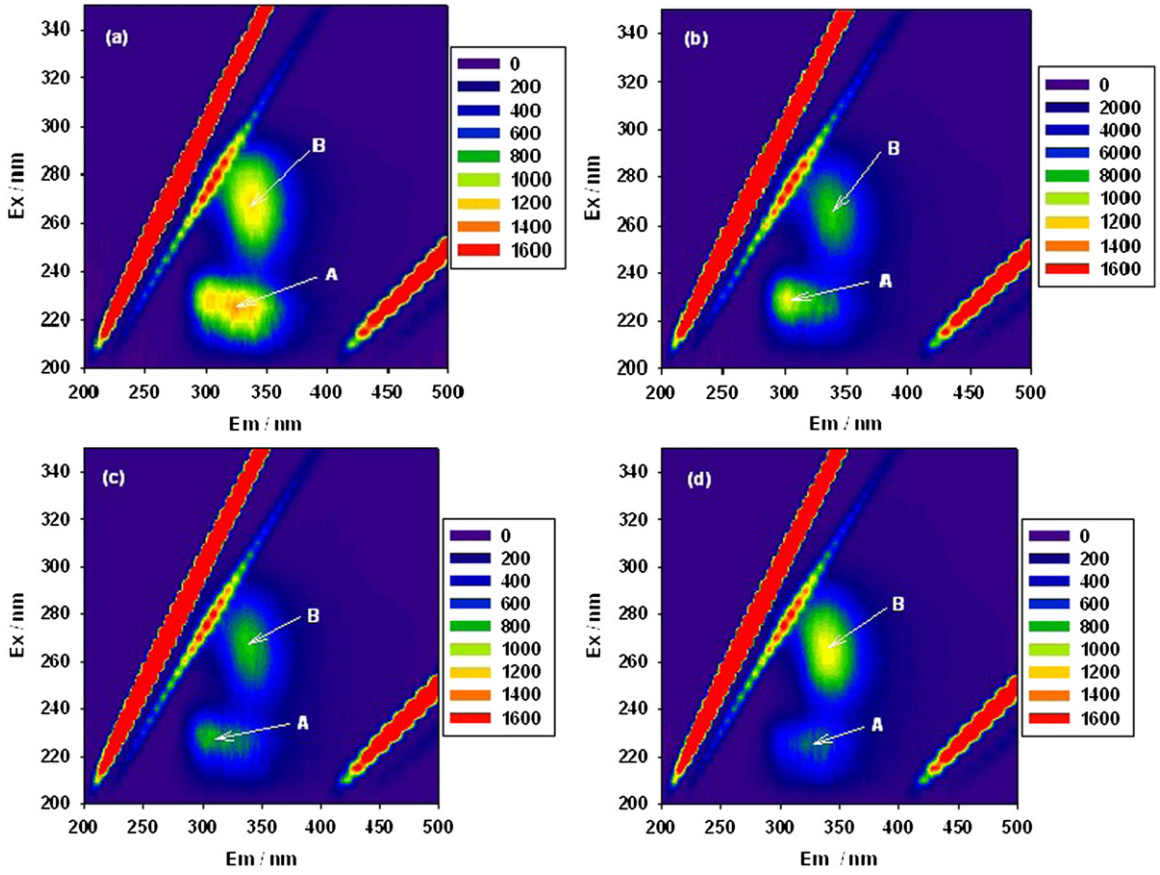

Fig. 1. Typical three-dimensional fluorescence EEM spectra of HSA at 35 °C in the absence (a) and presence of 0.5 mg/L Sb of three different forms (b) antimony potassium tartrate; (c) antimony trichloride; (d) potassium pyroantimonate).

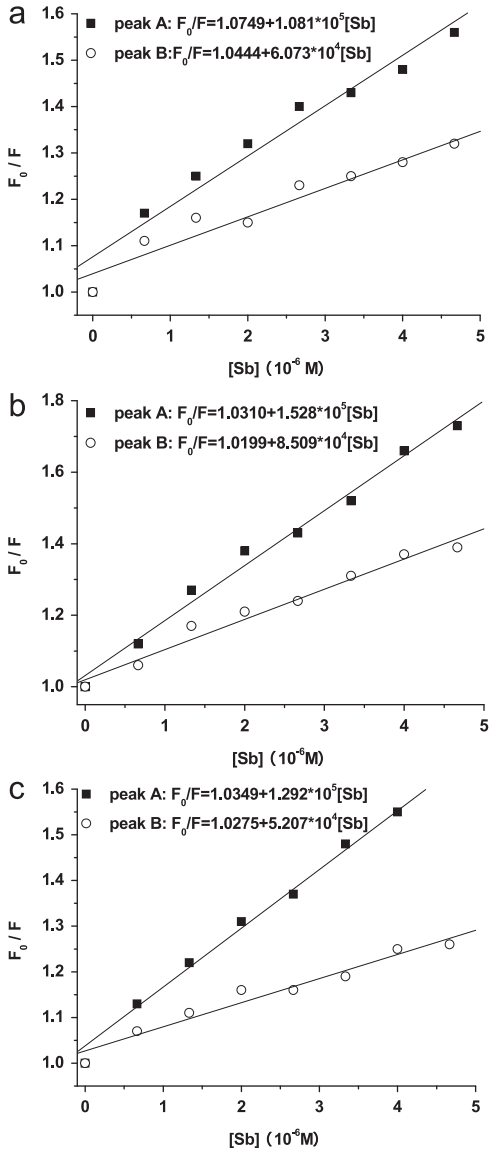

Fig. 2. The Stern–Volmer plots of fluorescence quenching of HSA titrated with Sb solution. (a) Antimony potassium tartrate. (b) Antimony trichloride. (c) Potassium pyroantimonate.

the EEM spectra of HSA, Peak A (Ex/Em: 225/326–334) and peak B (Ex/Em: 265–270/336–340) were attributed to aromatic-like and tryptophan-like fluorophores, respectively [28]. Peak A mainly exhibits the fluorescence spectra behavior of polypeptide chain backbone structures. Peak B reveals the spectra feature of tryptophan residues [29]. The wavelength (Ex/Em) of fluorescence peaks (A and B) used for quenching calculations of fluorescence intensity were 225/326 (A) and 270/336 (B) for HSA-antimony potassium tartrate system, 225/334 (A) and 270/340 (B) for HSA-antimony trichloride system, and 225/330 (A) and 265/340 (B) for HSA-potassium pyroantimonate system. The fluorescence intensity of both peak A and B decreased after addition of Sb, indicating that Sb interacts with HSA and thus quenches its fluorescence. The decrease amplitude of fluorescence intensity of the two peaks was different for various forms of Sb. Addition of 0.5 mg L^{-1} antimony potassium tartrate, antimony trichloride and potassium pyroantimonate reduced the fluorescence intensities of peak A by 36.0%, 42.1% and 37.9%, respectively and the fluorescence intensity of peak B by 24.1%, 28.2% and 20.4%, respectively. Pollutants can lead to conformation change of HSA and this might be the reason for explaining the decrease of fluorescence intensity of fluorophores in HSA after addition of Sb [30]. The initial HSA solution pH was 7.4 and addition of Sb had little effect on solution pH. pH was 7.39 for HSA-antimony potassium tartrate system, and 7.41 for both HSA-antimony trichloride and HSA-potassium pyroantimonate systems. This means fluorescence quenching is not due to change of solution pH but due to Sb compounds.

3.2. The fluorescence quenching

Fig. 2 showed that within the tested Sb concentration range, the change of fluorescence intensity agreed well with the Stern–Volmer equation. The linear Stern–Volmer plot generally indicates a class of fluorophore that are equally accessed by the quencher [31]. The fluorescence quenching process can be dynamic quenching, which is due to collision between the fluorophore and quencher, or static quenching due to the complexation between the fluorophore and the quencher. Fluorescence quenching was described by the Stern–Volmer Eq. (1) [31].

$$\frac{F_0}{F} = 1 + k_q \tau_0 [\mathrm{Sb}] = 1 + K_{sv} [\mathrm{Sb}] \quad (1)$$

where, F_0 and F represent the fluorescence intensity in absence and presence of quencher, respectively. K_q is the quenching rate constant, K_{sv} is the quenching constant, τ_0 is the average lifetime

Table 1
The Stern–Volmer fluorescence quenching constants K_{sv} of HSA by different substances.

T (K)	Substances	Quenching constants K_{sv}($\times 10^5$/M)	References
308	Antimony potassium tartrate	1.08 (peak A, R^2=0.95), 0.61 (peak B, R^2=0.93)	This study
308	Antimony trichloride	1.53 (peak A, R^2=0.99), 0.85 (peak B, R^2=0.98)	This study
308	Potassium pyroantimonate	1.29 (peak A, R^2=0.99), 0.52 (peak B, R^2=0.96)	This study
308	Chromium(VI)	0.15	[14]
308	APT	0.14	[15]
308	Copper based drug	1.89	[16]
310	Rhaponticin	2.15	[17]
310	Theasinesin	0.77	[18]
310	Costunolide	0.07	[19]
310	Dehydrocotus lactone	0.06	[19]
310	Fullerol	0.38	[20]
312	5-Iodouridine	0.14	[21]
306	BMPT	0.17	[22]
298	Amlodipine	0.83	[23]
298	Uranium(VI)	0.13	[24]

Table 2
The modified Stern–Volmer model parameters for HSA-Sb and HSA-Fullerol system.

T (K)	Substances	Condition stability constants K_a ($\times 10^5$/M)	binding capacities (f)	References
308	Antimony potassium tartrate	8.13 (peak A, R^2=0.98), 9.12 (peak B, R^2=0.98)	0.42 (peak A) 0.28 (peak B)	This study
308	Antimony trichloride	2.51 (peak A, R^2=0.99), 4.27 (peak B, R^2=0.96)	0.80 (peak A) 2.32 (peak B)	This study
308	Potassium pyroantimonate	3.63 (peak A, R^2=0.99), 9.77 (peak B, R^2=0.97)	0.56 (peak A) 0.13 (peak B)	This study
310	Fullerol	0.12	–	[20]

of the fluorescence in absence of quencher, which is taken as 10^{-8} s [31], and [Sb] is the Sb concentration.

The values of K_{sv} and R^2 were summarized in Table 1. All K_q values for binding of three forms of Sb to HSA were two orders of magnitude bigger than the maximum diffusion collision quenching rate constant (2.0×10^{10}/M/s) for a variety of quenchers with biopolymers [32], indicating the fluorescence quenching process was mainly governed by static quenching by formation of complex. Like many most other quenching substances [13–24] (Table 1), Sb significantly quenched the fluorescence of HSA, indicating the strong complexing ability of HSA toward Sb.

In order to understand more about the quenching mechanism, the fluorescence quenching data were further analyzed by the modified Stern–Volmer Eq. (2):

$$\frac{F_0}{(F_0-F)}=\frac{1}{(fK_a[\mathrm{Sb(III)}])}+\frac{1}{f} \tag{2}$$

where f is the fraction of the initial fluorescence corresponding to the binding fluorophore and K_a the condition stability constant.

In this modified equation, both the quenchable fluorophores and the quencher inaccessible fluorophores were considered. Following Lakowicz [31], the total fluorescence (F_0) fluorophores in absence of quencher, F_0, equals to F_{0a} and F_{0b}.

$$F_0=F_{0a}+F_{0b} \tag{3}$$

where F_{0a} is the fluorescence of the fluorophore moieties that can complex with quencher and F_{0b} is the fluorescence of the inaccessible fluorophore moieties.

In the presence of quencher, only the F_{0a} will change and the observed fluorescence intensity will be provided to form the modified Stern–Volmer equation to evaluate the complexation parameters, i.e., conditional stability constants and binding capacities.

$$f=\frac{F_{0a}}{(F_{0a}+F_{0b})} \tag{4}$$

All the titration data were well fitted with the modified Stern–Volmer equation (R^2=0.96–0.99). f and K_a for peaks A and B in the presence of various forms of Sb were calculated and summarized in Table 2.

The f and K_a of tryptophan-like fluorophores of peak B differed more significantly among the three forms of Sb than those of peak A. In the presence of antimony trichloride, the values of f for both peaks were highest, indicating that the fraction of fluorescent components accessible to antimony trichloride was maximal. However, the lowest values of K_a for HSA-antimony trichloride system showed relatively weak stability of HSA-antimony trichloride complexes. Values of K_a for HAS-Sb complexes were far bigger than that of HSA-fullerol complex [20] (Table 2). Interaction of antimony potassium tartrate and potassium

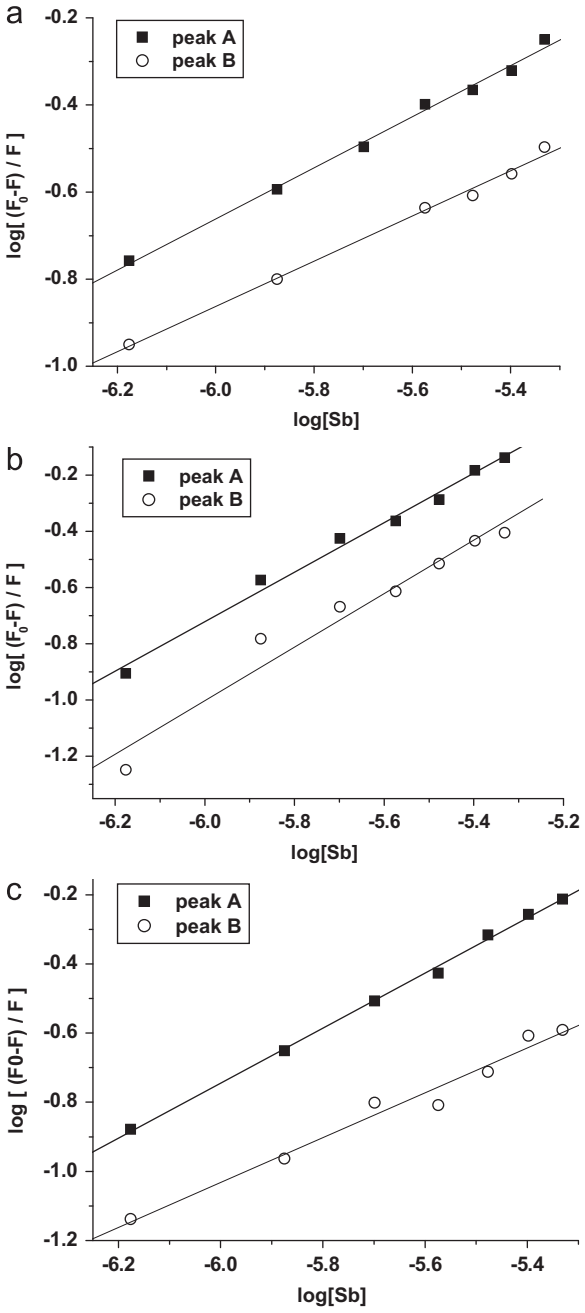

Fig. 3. The plots log[(F_0-F)/F] versus log[Sb] for HSA titrated with different forms of Sb. (a) Antimony potassium tartrate. (b) Antimony trichloride. (c) Potassium pyroantimonate.

Table 3
The binding constants (K_b) and binding sites (n) for HSA complexing with different substances.

Temperature (K) and substances		Binding constant K_b($\times 10^4$/M)	Binding site (n)	References
308	Antimony potassium tartrate	0.07 (peak A, R^2=0.99), 0.02 (peak B, R^2=0.99)	0.59 (peak A), 0.52 (peak B)	This study
308	Antimony trichloride	3.55 (peak A, R^2=0.99), 5.01 (peak B, R^2=0.96)	0.88 (peak A), 0.95 (peak B)	This study
308	Potassium pyroantimonate	1.08 (peak A, R^2=0.99), 0.07 (peak B, R^2=0.98)	0.80 (peak A), 0.65 (peak B)	This study
308	Chromium(VI)	1.68	3.96	[14]
308	Copper based drug	1.94	1.24	[16]
310	Rhaponticin	9.7	0.93	[17]
310	Theasinesin	0.22	0.98	[18]
310	Costunolide	0.92	0.98	[19]
310	Dehydrocostus lactone	0.76	1.00	[19]
312	5-Iodouridine	0.57	–	[21]
306	BPMT	2.61	0.80	[22]
298	Amlodipine	36.3	–	[23]
298	Propranolol	2.29	–	[23]
298	Co(II)	9.3	–	[37]
298	Perfluorooctane sulfonate	2.2	–	[38]
298	Perfluorooctanoic acid	27	–	[38]
310	1-naphthol (1N)	1.03	–	[39]
310	2-naphthol (2N)	1.49	–	[39]
310	8-quinolinol (8H)	0.19	–	[39]

pyroantimonate with HSA formed more stable complexes, suggesting that antimony potassium tartrate and potassium pyroantimonate might have more persistent toxicity to human blood system.

3.3. The binding constant and binding sites

For ligand molecules that bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the Hill equation [33]:

$$\log\left(\frac{(F_0-F)}{F}\right) = \log K_b + n\log[\mathrm{Sb}] \quad (5)$$

where K_b is the binding constant and n the number of binding sites. The binding constants (K_b) can reflect the interactive intensity between fluorophore and quencher. The greater of the binding constant is, the stronger of binding capacity of fluorophore is. The values of n express the binding sites provided by fluorophore to quencher molecule.

It was observed that plots log $[(F_0-F)/F]$ versus log [Sb] for HSA titrated with different forms of Sb showed good linearity (Fig. 3). The binding constant (K_b) and the number of binding site (n) of the HSA-Sb system were listed in Table 3. The binding site numbers, n, in HSA-antimony trichloride complex for all peaks and in HSA-potassium pyroantimonate complex for peak A were approximately equal to 1, which implied that there was one independent class of binding site for Sb towards HSA. The value of n in HSA-potassium pyroantimonate complex for peak B and HSA-antimony potassium tartrate complex for all peaks were less than 1, which suggested the negative cooperativity between multiple binding sites and the presence of more than one Sb binding site [34]. The n and K_b differed significantly with Sb forms. The values of n and K_b for complexation of HSA with antimony trichloride were highest, indicating that the binding ability of HSA to antimony trichloride was maximal. Table 3 summarized the binding constants and binding site numbers for interaction of HSA with some organic acids, drugs and metal ions in the literature. The values varied greatly depending on the quenchers. Most of previous studies focused on the interaction of HSA with drugs [14–16], lipids [19], organic acids [35] and some non-persistent organic pollutants [36]. Few studies were carried out on the interaction of HSA with heavy metals [12,37]. The binding ability of HSA to different substances was significantly affected by experimental condition, especially temperature [12,14–16]. In a temperature range from 306 to 310 K, the binding constant of HSA-antimony trichloride system was much higher than those of some HSA-drug system [14,16], HSA-lipid system [19] and HSA-Cr(VI) system [14]. However, HSA had stronger binding ability to some drugs, such as rhaponticin than antimony [17].

HSA has strong binding ability to Sb and the complexation might affect the retention time and metabolic pathway of Sb in body. The distribution of Sb in body was relevant to the valence-state of Sb. In hamsters, the levels of Sb(III) increase more rapidly in the liver than Sb(V), while the Sb(V) was predominantly uptake by skeleton. More Sb(III) was found in red blood cells than Sb(V) [9]. It was observed that human organ has different adsorption ability to different forms of Sb [38]. Some quantitative information on the adsorption of Sb was observed for some forms, ICRP has recommended 10% for antimony tartrate and 1% for all other forms of Sb as reference values for gastrointestinal absorption in humans. In this study, the binding ability of HSA to different forms of Sb followed the order of antimony trichloride > potassium pyroantimonate > antimony potassium tartrate. This result might be used to explain the difference in the toxicity of various forms of Sb to human body [1].

4. Conclusions

The binding interaction between HSA and three forms of antimony were investigated by EEM spectroscopy. It was found that antimony can effectively quench the fluorescence intensity of HSA. HSA had ability to complex different forms of Sb with the following order: antimony trichloride > potassium pyroantimonate > antimony potassium tartrate. However, the stability of complexation between fluorescent components in HSA and antimony trichloride was relatively weak, whereas the complexation between fluorescent components and potassium pyroantimonate and antimony potassium tartrate are relatively stable. The data might illustrate the difference in the toxicity of various forms of antimony to human body and provide some quantitative information for the future study of molecular toxicology of this environmentally important heavy metal.

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