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ORIGINAL PAPER

Synchronous fluorescence determination and molecular modeling of 5-Aminosalicylic acid (5-ASA) interacted with human serum albumin

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Abstract In this paper, we proposed a new method for the determination of either human serum albumin (HSA) or 5-Aminosalicylic acid (5-ASA) by synchronous fluorescence spectra and examined the interaction between them using the molecular modeling method under simulative physiological conditions. The optimum conditions of synchronous fluorometric determination of HSA were investigated and the method was successfully applied to the determination of 5-ASA added to serum, urine, and saliva samples. The linear range of the determination of HSA and 5-ASA were 1.60–414 μg mL^{-1} and 0.76–22.95 μg mL^{-1}, the detection limits were 0.552 μg mL^{-1} and 0.38 μg mL^{-1}, respectively. In addition, the effect of various common ions on the determination of HSA with 5-ASA was also discussed at room temperature.

Keywords 5-Aminosalicylic acid (5-ASA) · Determination · Human serum albumin · Molecular modeling · Synchronous fluorescence spectrum

Introduction

The determination of proteins is very important in clinical medicine, biochemistry, and laboratory tests. The most frequently used methods are the biuret [1], bromocresol green (BCG) [2], Coomassie brilliant blue (CBB) [3], and bromophenol blue [4]. In recent years, novel methods such as spectrophotometry [5], spectrofluorimetry [6], RLS [7], and chemiluminescence [8] were developed. The most quantitative analysis for proteins is generally based on their fluorescence enhancement effect on organic dyes. However, the organic fluorophores often suffer from photobleaching, low signal intensities, and random on/off light emission (blinking) [9]. Photobleaching is usually caused by sudden decomposition of the emitter; it is the main factor limiting the maximum number of photons obtained from a fluorophore. Low signal intensities reduce the accuracy of determination. Intermittent light emission causes problems in real-time studies of biomolecular dynamics such as protein folding, signal transduction, and enzymatic catalysis.

Serum albumins are the most abundant proteins in plasma constituting 52% of the protein composition in this matrix. As the major soluble protein constituents of circulatory system, they possess many physiological functions and play a key role in the transport of many endogenous and exogenous ligands. Human serum albumin (HSA) is the most important and abundant constituent of blood plasma and serves as a protein storage component. HSA serves as a transport carrier for a variety of small species, such as fatty acids, cations and many diverse drugs [10, 11], present in the systematic circulation, due to its very unique single-polypeptide globular multidomain structure [12].

In this study, the synchronous fluorescence technique was applied to determine 5-ASA and HSA. Synchronous fluorescence scan (SFS) analysis has become a new attractive method for the determination of biomolecules [13] since its introduction by Lioyd and Evett [14]. In the

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synchronous spectra [15], the sensitivity associated with fluorescence is maintained while offering several advantages: spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects. Because of its sharp, narrow spectrum, SFS serves as a very simple, effective method of obtaining data for quantitative determination in a single measurement [16].

5-Aminosalicylic acid (5-ASA) (Scheme 1) [17, 18] is a drug for treatment of ulcerus colonitis and enteritis, with the excellence of high efficiency and low toxicity. In our previous work [19], we investigated the binding mechanism between 5-ASA and HSA and suggested that hydrophobic interaction was the predominant intermolecular forces stabilizing the complex, which was in good agreement with the results of molecule modeling study in this work. However, the investigation on the mutual determination of 5-ASA and HSA by fluorescence spectra has not been reported at the molecular level.

In this work, a novel method as 5-ASA a fluorescence probe for the mutual quantitative determination between 5-ASA and HSA using synchronous fluorescence technique in combination with molecular modeling method at room temperature under the simulative physiological conditions was proposed. The results obtained from fluorescence spectra and modeling verified the formation of a new complex between 5-ASA and HSA. Based on the binding of 5-ASA with HSA, the synchronous fluorescence technique was successfully employed to determine 5-ASA and HSA in serum, urine and saliva samples with a wide linear range and satisfactory results.

Experimental

Apparatus

Fluorescence measurements were carried out on a FP-6200 spectrofluorimeter (JASCO, Japan) equipped with 1.0 cm quartz cells and a thermostat bath. pH measurements were made with a pHS-3 digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode.

Reagents

HSA (Sigma) was directly dissolved in water to prepare stock solution (4.0×10^{-5} mol L^{-1}) and then stored at 0–4 °C. 5-ASA 1.0×10^{-3} mol L^{-1}, 0.1 mol L^{-1} Tris-HCl buffer solution of pH 7.4 and 0.5 mol L^{-1} sodium chloride; Appropriate amount of common ions, serum and urine samples were prepared. Human serum sample was obtained from the Hospital of Henan Normal University, the serum and urine samples had been diluted 100-fold with double distilled water before determination. All reagents were of analytical reagent grade and double distilled water was used for all the measurements.

Procedures

To a 10 mL comparison tube, 2 mL of sodium chloride solution, 2 mL Tris-HCl buffer, appropriate amount of 5-ASA and HSA were added and were diluted to 10 ml with water. Fluorescence spectra were obtained at excitation and emission wavelengths of 290 and 300–550 nm, respectively and with $\Delta\lambda$=20 nm. Synchronous fluorescence spectra were obtained at wavelengths of 280–350 nm.

Molecular modeling study

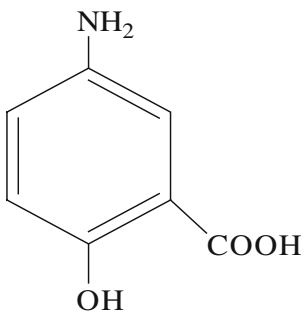
The potential energy of the three-dimensional structure of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were generated by molecular modeling software Sybyl 6.9.1 [20]. The geometries of 5-ASA were subsequently optimized using the Tripos force field with Gasteiger-Marsili charges. The AutoDock3.05 [21, 22] program was used to calculate the interaction modes between the drug and HSA. Lamarckian genetic algorithm (LGA) implemented in Autodock was applied to calculate the possible conformation of the drug that binds to protein. During docking process, a maximum of 10 conformers was considered for this compound. The conformer with the lowest binding free energy was used for further analysis. All calculations were performed on SGI FUEL workstation.

Results and discussion

Fluorescence spectra

Fluorescence quenching of the single tryptophan residue in HSA was used to measure drug-binding affinity [23]. Tryptophan fluorescence is the most frequently examined among the three intrinsic aromatic fluorophores in HSA molecules to obtain information about conformational

Scheme 1 The structure of 5-ASA



changes. The effect of 5-ASA on HSA and the conformation changes of HSA were evaluated by measuring the intrinsic fluorescence intensity of protein tryptophan residues in the absence and presence of 5-ASA. The addition of 5-ASA caused a dramatic decrease in the fluorescence emission intensity of HSA with a conspicuous change in the emission spectra (Fig. 1). HSA shows a strong fluorescence emission with a peak at 341 nm on excitation at 290 nm due to its single Tryptophan residue (Trp-214), while 5-ASA has strong fluorescence at 501 nm on excitation at 323 nm under the present experiment conditions. With gradual increase in drug concentrations, the maximum emission wavelength were shifted from 341 to 352 nm. It can be seen that a higher excess of 5-ASA led to more effective quenching of the chromophore molecules fluorescence. The strong quenching of the fluorescence clearly indicated that the binding of the drug to HSA changed the microenvironment of tryptophan residue and the tertiary structure of HSA. For 5-ASA, it caused a concomitant increase in the fluorescence emission around 501 nm, which is the characteristic wavelength of the bound-HSA. This phenomenon might be the result of the radiations energy transfer between 5-ASA and HSA. Therefore, the obvious enhancement of fluorescence intensity and the change of fluorescence spectra verified the formation of a new complex between 5-ASA and HSA.

Modeling

The complementary applications of molecule modeling have been employed by computer methods to improve the understanding of the interaction of 5-ASA and HSA. Descriptions of the 3-D structure of crystalline albumin have revealed that HSA comprises three homologous domains, (I-III): I (residues 1–195), II (196–383), III (384–585) [12], each domain is a product of subdomains that possess common structural motifs. The crystallographic analysis reveals that the principal regions of ligand binding to HSA are located in hydrophobic cavities in subdomains IIA and IIIA, respectively, which exhibit similar chemistry [11]. Despite very high stability, HSA is a flexible protein with the 3D structure susceptible to environmental factors such as pH, ionic strength, etc. [24]. It was proposed that the binding took place near Trp-214 and led to a conformational change with a local perturbation of the IIA binding site in HSA [25, 26]. There is a large hydrophobic cavity present in subdomain IIA to which many drugs can bind [25].

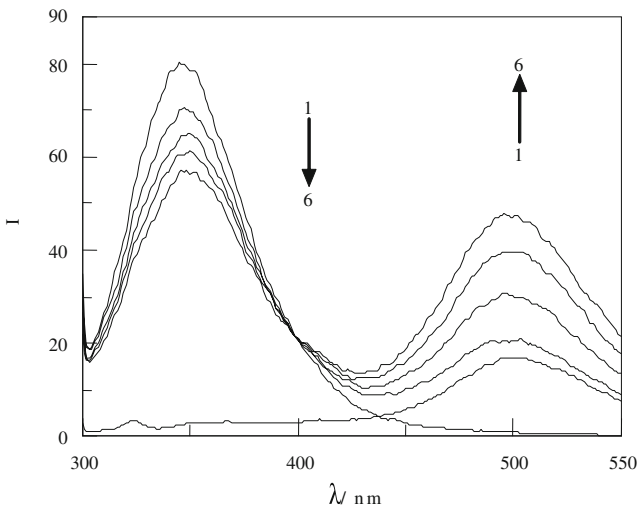


Fig. 1 Fluorescence spectra of 5-ASA-HSA $C_{HSA} = 4 \times 10^{-5}$ mol L^{-1}; from 1–6, $C_{5-ASA} = 0, 0.1 \times 10^{-4}$, 0.2×10^{-4}, 0.3×10^{-4}, 0.4×10^{-4}, 0.5×10^{-4} mol L^{-1}

The crystal structure of HSA in complex with warfarin was taken from the Brookhaven Protein Data Bank (entry codes 1h9z). The potential of the 3-D structure of HSA was assigned according to the Amber 4.0 force field with Kollman-all-atom charges. The initial structures of all the molecules were generated by molecular modeling software Sybyl 6.9.1 [20]. The geometry of the molecule was subsequently optimized to minimal energy using the tripos force field with Gasteiger-Marsili charges. Then it was used to replace warfarin in the HSA-warfarin crystal structure. The Flexx program was applied to calculate the possible conformation of the ligands that bind to the protein. As drug-binding sites I and II of HSA are located in hydrophobic cavities in subdomain IIA and IIIA, respectively, which exhibit similar chemistry, despite very high stability, HSA is a flexible protein with the 3D structure susceptible to environment factors such as pH, ionic strength, etc. Therefore, the lowest root means square (RMS) was used for further analysis. Based on this type of approach, a computational model of the target receptor has been built, partial binding parameters of the 5-ASA-HSA system were calculated through SGI FUEL workstations. The best energy ranked results are shown in Fig. 2. As shown in Fig. 2, the salicylic acid moiety is located within the binding pocket. The ring of 5-ASA was inserted in the hydrophobic cavity of site I, and it is important to note that the residue ARG-218 and the trptophan residue of HSA (Trp214) are in close proximity to the ring of 5-ASA suggesting the existence of hydrophobic interaction between them. Further, this finding provides a good structural basis to explain the efficient fluorescence quenching of HSA emission in the presence of the 5-ASA. On the other hand, there are hydrogen interactions between 2-OH of 5-ASA and the residues ASP-451, ARG-218, ARG-222 and ALA-291 of HSA; another hydrogen bond is related to 1-COOH of 5-ASA. The results indicated that the formation of hydrogen bond decreased the hydrophilicity and increased the hydrophobicity to stabilize in the 5-ASA-

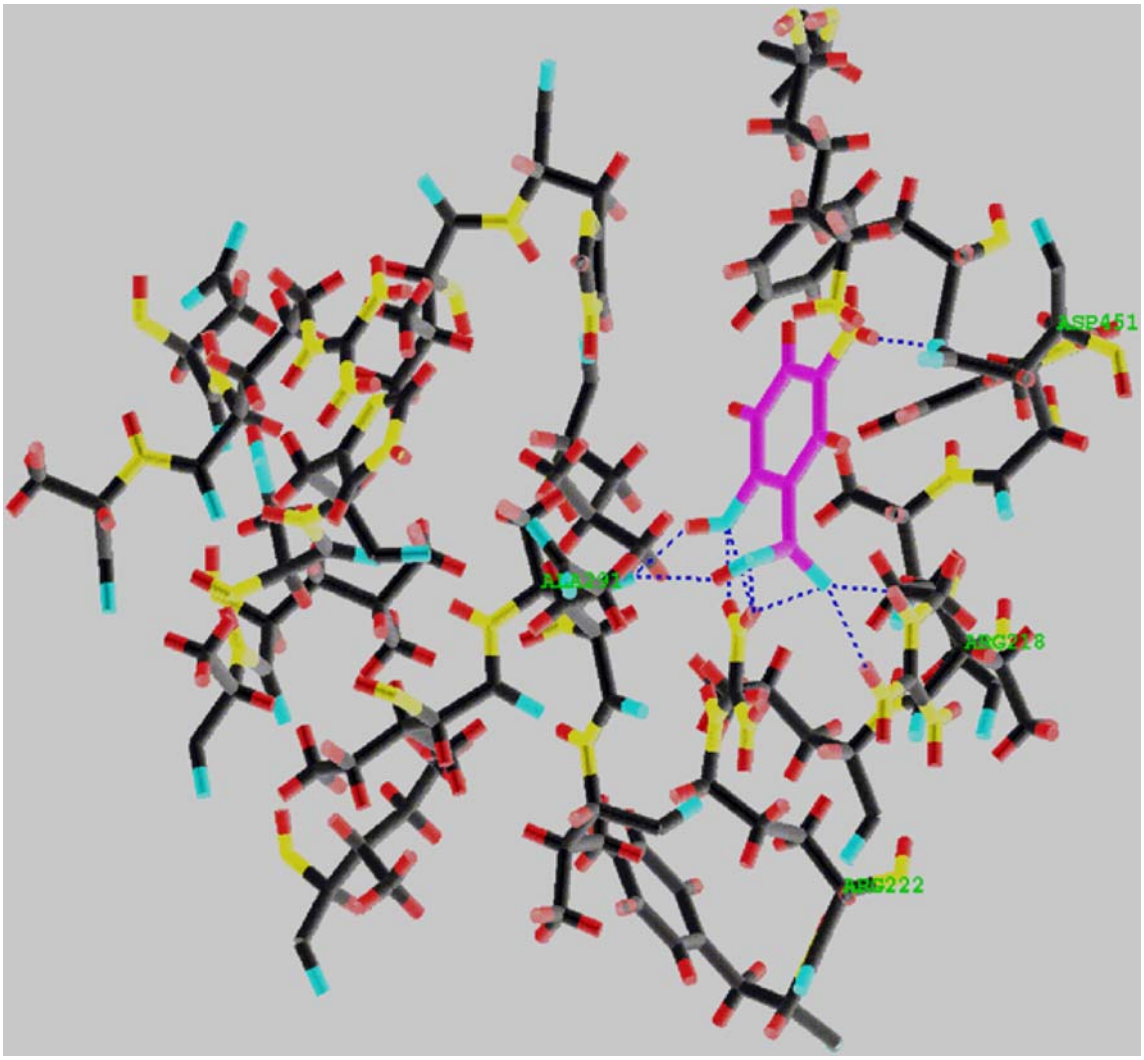


Fig. 2 The interaction mode between 5-ASA and HSA; only residues around 0.8 nm of 5-ASA is displayed. The residues of HSA and the 5-ASA structure are represented using different stick model. The hydrogen bond between 5-ASA and HSA is represented using the blue dashed line

HSA system. The amino acid residues with a benzene ring can match that of the 5-ASA in space in order to firm the conformation of the complex. In conclusion, the results obtained from modeling indicated that the interaction between 5-ASA and HSA is dominated by hydrophobic force, and there are also hydrogen bonds between the drug and the polar amino acid residues.

The experimental methods for the determination of protein and drug

Based on the binding of HSA to 5-ASA, we employed synchronous fluorescence spectra to quantitative determine the HSA and 5-ASA. The character of synchronous fluorescence spectra between 5-ASA and HSA was given in Fig. 3. The fluorescence intensity decreased gradually with the concentration of 5-ASA increasing.

Under the optimum conditions, the concentration of HSA was kept unchanged with different concentrations of the 5-ASA to determine 5-ASA by normal fluorescence at λ_{ex} 290 nm and λ_{em} 300–550 nm. The fluorescence quenching spectra of HSA at various concentrations of 5-ASA is shown in Fig. 4. HSA has a strong fluorescence emission band at 341 nm by fixing the excitation wavelength at 290 nm. Its fluorescence intensity decreases in the presence of 5-ASA and a very slight red shift was observed for the emission wavelength with increasing 5-ASA concentration, indicating that a 5-ASA-HSA complex was formed, which could quench the fluorescence of HSA and change the microenvironment of tryptophan residue. For 5-ASA, its fluorescence intensity increases in the presence of HSA and takes on a good linear range between them. These results suggested that there was strong interaction between 5-ASA and HSA, which was in

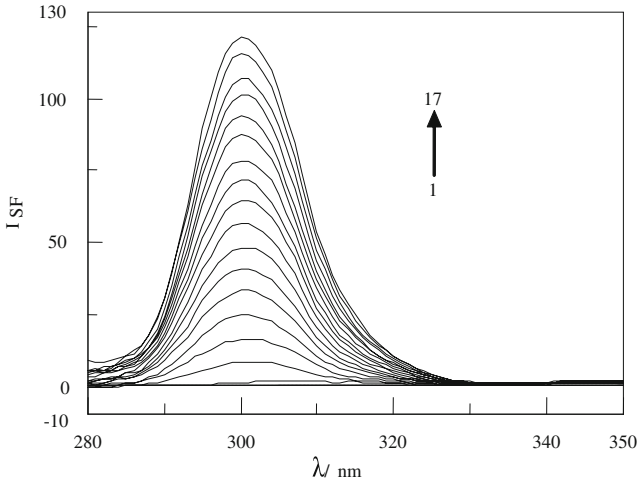


Fig. 3 Synchronous fluorescence spectra of HSA under the optimum conditions $C_{5\text{-ASA}}=10^{-3}$ mol L^{-1}; from 1–17, $C_{HSA} = 0, 0.4 \times 10^{-6}$, 0.8×10^{-6}, 1.2×10^{-6}, 1.6×10^{-6}, 2.0×10^{-6}, 2.4×10^{-6}, 2.8×10^{-6}, 3.2×10^{-6}, 3.6×10^{-6}, 4.0×10^{-6}, 4.4×10^{-6}, 4.8×10^{-6}, 5.2×10^{-6}, 5.6×10^{-6}, 6.0×10^{-6}, 6.4×10^{-6} mol L^{-1}

accordance with the results from binding mode in our study [19]. Based on the interaction between them, we determined 5-ASA with HSA successfully.

Optimization of experimental conditions for the determination of the protein and the drug

In order to select an optimized analytical system, various experimental parameters including medium, buffer solution, pH, and sequence of regents, amount of reagents were studied.

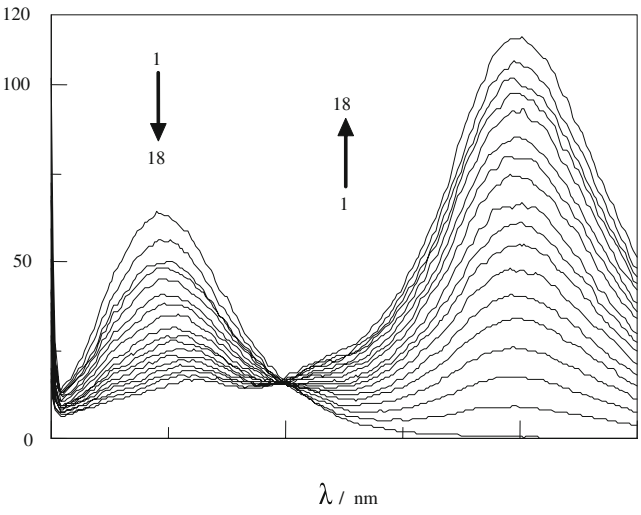


Fig. 4 Fluorescence spectra of 5-ASA-HSA under the optimum conditions $C_{HSA} = 4 \times 10^{-5}$ mol L^{-1}; from 1–18, $C_{5-HSA} = 0, 0.1 \times 10^{-4}$, 0.2×10^{-4}, 0.3×10^{-4}, 0.4×10^{-4},0.5×10^{-4}, 0.6×10^{-4}, 0.7×10^{-4}, 0.8×10^{-4}, 0.9×10^{-4}, 1.0×10^{-4}, 1.1×10^{-4}, 1.2×10^{-4}, 1.3×10^{-4}, 1.4×10^{-4}, 1.5×10^{-4}, 1.6×10^{-4}, 1.7×10^{-4} mol L^{-1}

Effect of media

Several buffer solutions (Tris-HCl, KH_2PO_4-$Na_2B_4O_7$, H_3BO_3+KCl-Na_2CO_3, $Na_2B_4O_7$-H_3BO_3, Na_2HPO_4-$C_6H_8O_7$) were tested in the present experiment. The sensitivity was higher in Tris-HCl buffer solution than in others. Therefore, Tris-HCl buffer solution was used in the experiment. The effect of its concentration on synchronous fluorescence intensity has been investigated in the range of 0.5–3.0 mL. It was found that the sensitivity was higher when 2.0 mL of buffer solution has been used.

The addition sequence of reagents

We tried out the effect of addition sequence of reagents on synchronous fluorescence intensity of system according to permutation and combination, and found that the sensitivity was higher if the order was NaCl→HSA→Tris-HCl→5-ASA.

The selection of the stocks shift (Δλ)

Under the optimum conditions described above, synchronous fluorescence spectra were recorded while Δλ was 5, 10, 15, 20, 30, 40, 50, 60 nm, respectively. It was found that the spectrum was more symmetrical if Δλ was 20 nm. In such circumstance, the synchronous fluorescence of protein (HSA) was caused mainly by tryptophan residue.

Effect of pH

The influence of pH was investigated in the range of 7.20–8.40. It was shown that the fluorescence intensity was

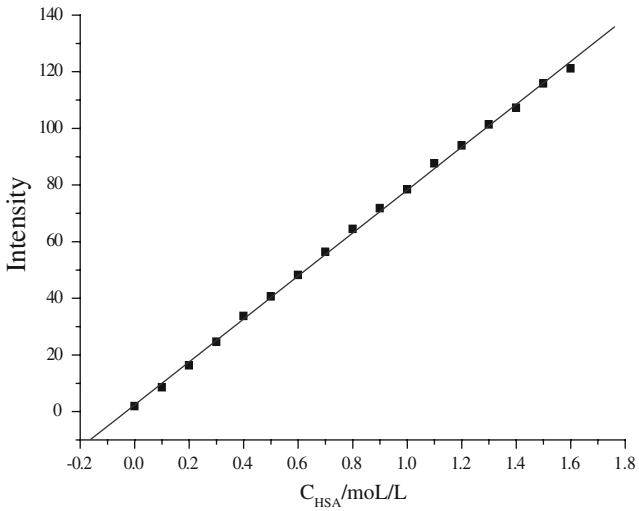


Fig. 5 The working curve of synchronous fluorescence for determination of HSA

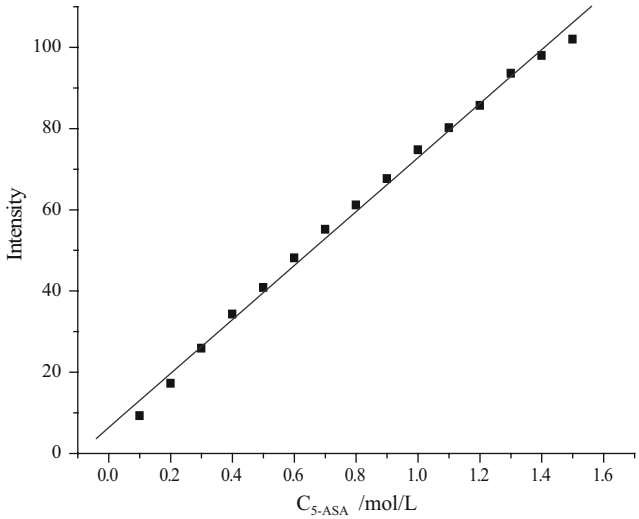


Fig. 6 The working curve of normal fluorescence for determination of 5-ASA

higher in pH 7.40 than in others, and also because the experiment was studied under physiological conditions. Therefore, Tris-HCl buffer solution at pH 7.40 was used in the experiment.

The effect of 5-ASA amount

The effect of 5-ASA amount was test in the range of 0.2–2.0 mL. It was found that the synchronous fluorescence intensity was highest when 0.2 mL of 5-ASA was added. However, if the amount of 5-ASA was too small, the sensitivity of the system would reduce; Therefore, 0.4 mL of 5-ASA was used in the experiment.

Table 1 The effect of common ions on the determination of human serum albumin

Coexisting substance	Interferential ratio	Error
K^+	43	4.87%
NH_4^+	33	5.15%
Na^+ (Cl^-)	40(66)	4.95%
SO_4^{2-}	6	3.74%
CO_3^{2-}	9	4.72%
PO_4^{3-}	18	4.56%
Zn^{2+}	3	5.14%
Ca^{2+}	35	5.58%
starch	2	4.29%
dextrine	24	−5.04%
dextrose	44	−4.86%
dl-lysine	2	−5.07%
fructose	25	−4.84%
serine	13	4.87%
dl-threonine	3	−4.64%

Table 2 Determination results of 5-ASA in samples (n=6)

Samples	Initial/μg mL^{-1}	Added/μg mL^{-1}	Found/μg mL^{-1}	Recovery/ %
Serum	0	3.06	3.08	100.7
	0	6.12	6.35	103.3
	0	9.18	9.45	102.9
Urine	0	1.53	1.54	100.6
	0	3.06	3.10	101.3
	0	4.59	4.76	103.7
Saliva	0	1.53	1.52	99.3
	0	3.06	3.08	100.7
	0	4.59	4.57	99.6

Precision, limit of detection and working curve

For the protein

Under the conditions described above, the working curve for the determination of HSA was shown in Fig. 5.

The linear range was 1.60–414 μg mL^{-1} for the determination of HSA (with 5-ASA), with the correlation coefficient of 0.9995. The linear equation was $I_{HSA} = 2.42 + 1.31 \times 10^7 C_{HSA}$. The detection limit, as defined by IUPAC [27], was determined to be 0.552 μg mL^{-1} HSA with 5-ASA, which was obtained for 11 replicate determination of 4.0×10^{-5} mol L^{-1} HSA solutions. The experimental results demonstrated that there were good linear relationship between 5-ASA and HSA.

For the drug

Under the conditions described above, the working curve for the determination of 5-ASA is shown in Fig. 6.

Table 3 Determination results of HSA in samples (n=6)

Samples	Added/g mL^{-1}	Found/μg mL^{-1}	Recovery/ %	RSD/ %
Serum	0	80.77	105.7	0.89
	55.2	143.77		1.8
	110.4	201.96	105.6	0.93
	165.6	253.0	102.7	0.5
Urine	0	9.46		0.76
	27.6	38.27	103.3	1.26
	55.2	66.54	102.9	0.54
	82.8	94.57	102.5	0.85
Saliva	0	121.5		0.76
	27.6	149.3	100.1	1.26
	55.2	176.2	99.7	0.54
	82.8	204.2	99.9	0.85

The linear range was 0.76–22.95 μg mL^{-1} for 5-ASA (with HSA), with the correlation coefficients 0.9976. The linear equation is $I_{5-ASA} = 6.39 + 1.66 \times 10^5 C_{5-ASA}$. The detection limit is determined to be 0.38 μg mL^{-1} 5-ASA with HSA, which was obtained for 11 replicate determination of 1.0×10^{-3} mol L^{-1} 5-ASA solutions. The experimental results demonstrated that there were good linear relationship between 5-ASA and HSA.

The effect of common ions

Under the optimum conditions described above, the influence of some common ions on the determination of 5-ASA and HSA was investigated. The relative standard deviations concluded in the range of −5.0%–5.0%, the experimental results are listed in Table 1.

Analysis of samples

Under the experimental conditions, the present method was applied to the determination of the total proteins in the human serum, urine and saliva samples. The urine samples were diluted 100-fold and the saliva samples were diluted 10-fold to be the working solution. The results are given in Tables 2 and 3. It could be concluded that the proposed method for the determination of protein was reliable, sensitive, simple, and practical in clinical and biochemical applications, with good precision.

Conclusions

In this report, HSA and 5-ASA were measured by fluorescence spectroscopy and modeling studies under physiological conditions. The results were very close to the clinical data obtained from hospital. Therefore, the proposed method has great potential for the sensitive and rapid determination of proteins in serum samples and the theoretical data could be a useful guide for efficient drugs analysis. The results showed that the present method was comparable with other methods in terms of sensitivity, rapid, simplicity, and linear range. This method might be expanded to application in biochemistry and clinic practice.

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