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Synchronous fluorescence determination of human serum albumin with methyl blue as a fluorescence probe

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Abstract

A new synchronous fluorescence scan analysis was developed for the determination of HSA with high sensitivity with a triphenylmethane acid dye methyl blue as a fluorescence probe. When $\Delta\lambda = 140$ nm, the synchronous fluorescence peak of methyl blue is located at 323 nm and the synchronous fluorescence intensity of the methyl blue is significantly increased in the presence of trace HSA due to the complex formed between methyl blue and HSA at pH 4.1. Under optimal conditions, the calibration graphs are linear over the range 0.03–266.0 and 266.0–665.0 $\mu g\,mL^{-1}$ for human serum albumin (HSA). Limit of determination were 0.03 $\mu g\,mL^{-1}$ for HSA. In the detection of HSA in human serum samples, this method gave values close the clinical data got from hospital.

Keywords: Human serum albumin; Synchronous fluorescence; Triphenylmethane acid dye; Methyl blue

1. Introduction

Determination of protein quantity in biological liquids is of great importance in biology and medicine [1] and dyes as fluorescent probes are successfully applied for this approach [2], such as ethidium bromide [3], eosin B and eosin Y [4], Coomassie brilliant blue [5], Nana orange and Albumin Blue [6]. A number of assays have been continuously reported in recent years, such as those based on spectrophotometric [7,8], fluorometric [9,10], electrochemical [11] and chemiluminescence methods [12]. Most of the widely used protein assays are spectrophotometric methods, however, they have some limitations of in terms of sensitivity, selectivity, stability and simplicity [13].

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 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 [14,15], present in the systematic circulation, due to its very unique single-polypeptide globular multidomain structure [16].

In this study, the synchronous fluorescence technique was applied to determine HSA. Synchronous fluorescence spectroscopy (SFS) technique was introduced by Lloyd [17]. In the synchronous spectra [18], 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 [19]. Lun Wang's group determined proteins at nanogram levels by synchronous fluorescence technique with novel composite nanoparticles [20,21] and composite nanoclusters [22]. There are few reports on determination of HSA by synchronous fluorescence method with triphenylmethane dyes as probe.

Methyl blue (its chemical structure is shown in Fig. 1) is a kind of triphenylmethane acid dyes that has act as biologic coloring agent and pH-indicator [23]. In this paper, the synchronous fluorescence method was applied to determination HSA with the triphenylmethane acid dye methyl blue as a fluorescence probe. It is because that methyl blue has some advantage. Methyl blue is biological dye, which shows innocuity. A research indicated methyl blue has strong bacteriostatic activity [24]. Moreover, it

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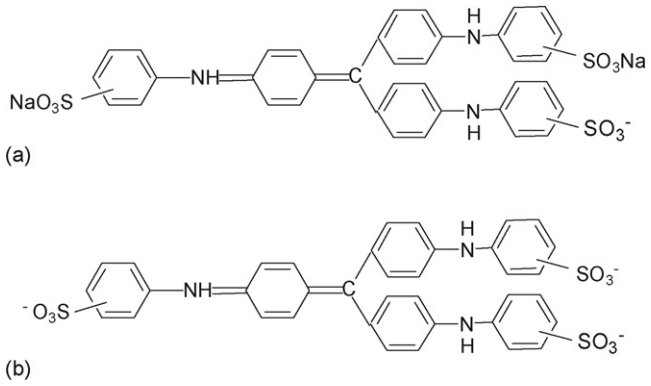

Fig. 1. The chemical structure of methyl blue. (a) Molecular form in acid solutions and (b) molecular form in basic solutions.

exists in two molecular forms in solution, the acidic form and basic form. It is sensitive to the change of pH value. So it is suitable for a variety of bimolecular application.

The synchronous fluorescence intensity of methyl blue is greatly enhanced in the presence of trace HSA. Based on this, a synchronous fluorescence method has been developed for the determination of HSA. Researches [20–22] reports that the linear range are 0.1–1.4, 0.1–10 and 0.03–2.0 $\mu g\,mL^{-1}$ for determination of HSA, respectively. Compared with those results, the method in our study has a wider linear range and satisfying limit of determination.

2. Experimental

2.1. Reagents and materials

Methyl blue (Third Reagent Factory of Shanghai, China) was used. The stock solutions of HSA (Sigma) was prepared by dissolving in 0.5% NaCl solution, and stocked at 0–4 °C. Britton–Robinson buffer of various pH values was prepared. All reagents were of analytical grade and used without further purification. Double distilled water was used throughout.

2.2. Apparatus

All fluorescence measurements were carried out on F-4500 fluorescence spectrophotometer (HITACHI, Japan), which was equipped with a 150-W xenon arc lamp, a recorder, dual monochromators and quartz cells (1 cm × 1 cm). A Shimadzu UV-265 double-beam spectrophotometer (Tokyo, Japan) equipped with 1.0 cm quartz cells was used for scanning the UV spectrum. All pH measurements were performed with a pHS-3C digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a glass electrode.

2.3. Experimental procedures

With Britton–Robinson buffer solution (pH 4.1), synchronous fluorescence spectra of methyl blue absence HSA and in the presence of various amounts of HSA were measured at $\lambda_{em} = 323$ nm ($\Delta\lambda = 140$ nm). We use the change of the relative synchronous fluorescence intensity of the system HSA.

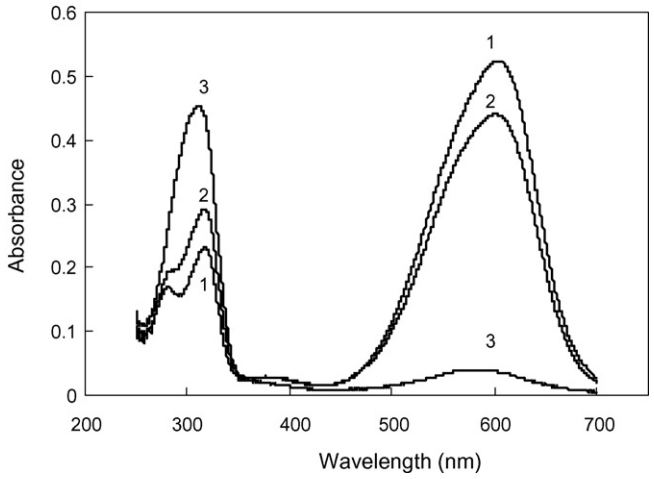

Fig. 2. Absorption spectrum of methyl blue: (1) pH 2.1; (2) pH 4.1; (3) pH 8.0.

3. Result and discussion

3.1. Spectra characteristics of methyl blue

Methyl blue exists in two molecular forms in solutions. The two molecular forms in acid and basic solutions have been introduced in Fig. 1 and the spectra of these forms are depicted in Fig. 2. The synchronous fluorescence peak of the methyl blue is located at 323 nm when the $\Delta\lambda = 140$ nm. The synchronous fluorescence spectra of methyl blue binding with various amounts of HSA can be seen in Fig. 3. This is because that triphenylmethane acid dye methyl blue exists in the acidic form in the medium of pH 4.1 and its $-SO_3-$ can combine with $-^+NH_3-$ of HSA to form a complex. The absorption spectrum of methyl blue

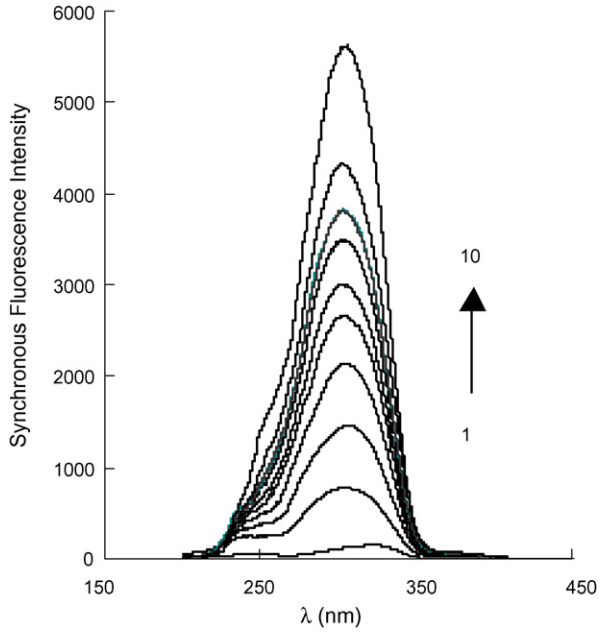

Fig. 3. The synchronous fluorescence spectrum of methyl blue in the presence of HSA of increasing concentration (from 1 to 10): [methyl blue] = 1.0×10^{-5} mol L^{-1}, [HSA] = 1: 0, 2: 49.9, 3: 99.8, 4: 133.0, 5: 166.3, 6: 199.5, 7; 232.8, 8: 266.0, 9: 399.0, 10: 665.0 $\mu g\,mL^{-1}$, $\Delta\lambda = 140$ nm, pH 4.1.

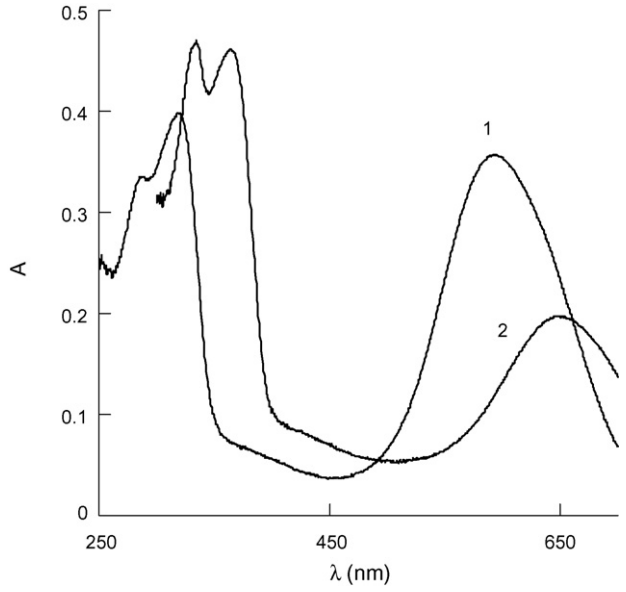

Fig. 4. Absorption spectrum of methyl blue absence and in the presence of HSA: [methyl blue] = 1×10^{-5} mol L^{-1}; [HSA] = 1: 0, 2: 4.0×10^{-6} mol L^{-1}; pH 4.1.

absence and in the presence of HSA suggested that the complex was formed between methyl blue and HSA (shown in Fig. 4).

The synchronous fluorescence intensity of methyl blue is significantly enhanced by the addition of HSA. The blank intensity is about nine at 453 nm when obtained by normal fluorescence (λ_{ex} = 314 nm), while that obtained by synchronous fluorescence is nearly zero at λ_{em} = 323. These results above indicate that the methyl blue could be used as a new fluorescence probe for sensitive determination of HSA.

3.2. Effect of the dye concentration

The concentration of methyl blue may have influence on the synchronous fluorescence intensity of the studied system (Fig. 5). The synchronous fluorescence intensity increased very slightly when the concentration of the dye was in the range of 1.0×10^{-5} to 3.0×10^{-5} mol L^{-1}. In order to get a high sensitivity, the concentration of 1.0×10^{-5} mol L^{-1} was recommended in this assay. The maximal molar absorptivity value of the dye in the visible $\varepsilon_{max} = 4.4 \times 10^4$ L (mol cm)$^{-1}$.

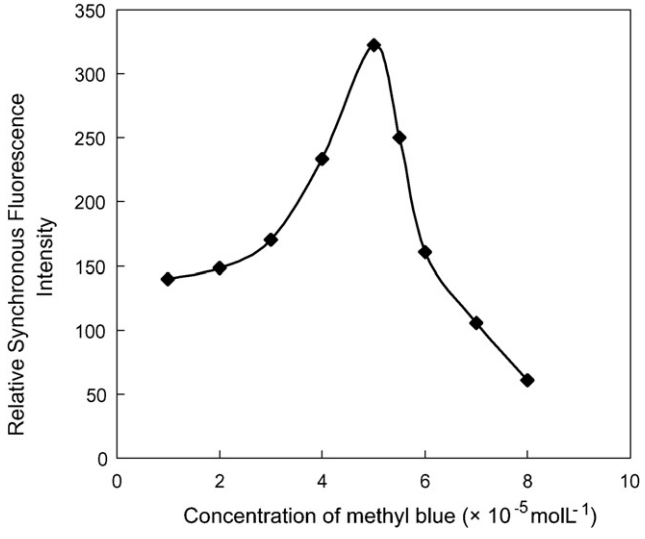

Fig. 5. Effect of concentration of methyl blue solutions.

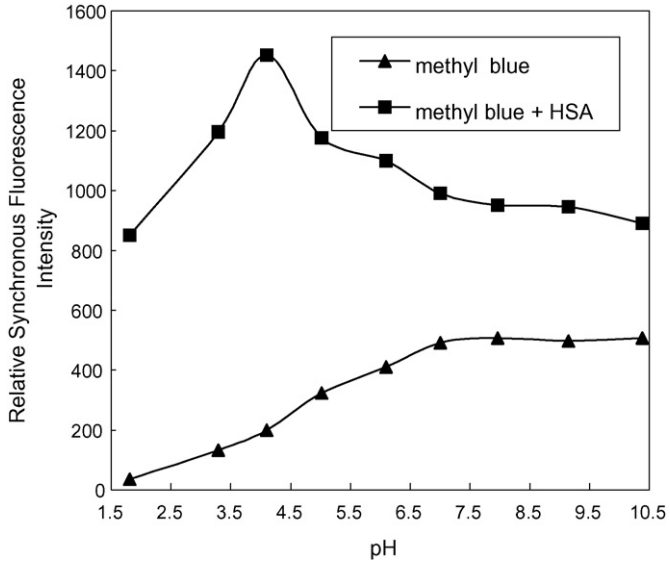

Fig. 6. Effect of pH value. [methyl blue] = 1×10^{-5} mol L^{-1}; [HSA] = 49.9 μg mL^{-1}.

3.3. Effect of pH and temperature

The influence of pH on the synchronous fluorescence intensity of the dye and the enhancement by HSA was examined over the pH range 1.8–10.5, as shown in Fig. 6. The maximum relative synchronous fluorescence intensity was occurred when pH is 4.1. So, we selected pH 4.1 as the suitable pH value in this assay. We use Britton–Robinson buffer to control the pH values. The maximum synchronous fluorescence intensities were reached when the mixed solutions had reacted at 20 °C for 10 min. The synchronous fluorescence remained for more than 1 h. So, 10 min and 20 °C were adopted in this assay.

3.4. Characteristics of synchronous fluorescence method

The synchronous fluorescence spectral was obtained by scanning simultaneously the excitation and emission monochromators. Thus, the synchronous fluorescence applied to the equation of synchronous luminescence [25]:

$$I_{SF} = kcdE_{ex}(\lambda_{em})E_{em}(\lambda_{ex} + \Delta\lambda) \quad (1)$$

where I_{SF} is the relative intensity of synchronous fluorescence, E_{ex} the excitation function at the given excitation wavelength, E_{em} the normal emission function at the corresponding emission wavelength, c the analytical concentration, d the thickness of the sample cell and k is the characteristic constant comprising of the "instrumental geometry factor" and related parameters. Since, the relationship of the synchronous fluorescence inten-

Table 1
Analytical parameters for HSA

Human serum sample	Standard regression equation (C, μg mL^{-1})	Regression coefficient	Linear range (μg mL^{-1})	Determination limit (μg mL^{-1})
	14.30C − 11.0	0.9974	0.03–266.0	0.03
	4.95C + 2307.2	0.9911	266.0–665.0	

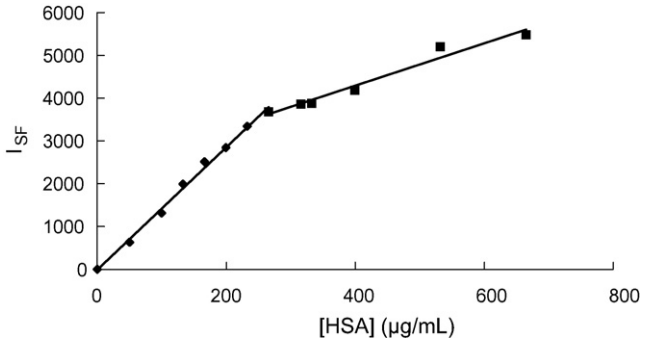

Fig. 7. The working curve of synchronous fluorescence method for determination of HSA: [methyl blue] = 1.0×10^{-5} mol L^{-1}; [HSA] from 0 to 665.0 μg mL^{-1}, Δλ = 140 nm; pH 4.1.

sity (I_{SF}) and the concentration of HSA should follow the I_{SF} equation, I_{SF} should be in direct proportion to the concentration of HSA.

3.5. Precision, limit of detection and working curve

The calibration graph for HSA under the optimum conditions is shown in Fig. 7 and the analytical parameters for HSA are in Table 1. The highest sensitivity in the experiment is reached when the maximum amount of methyl blue is bound to HSA at a concentration of 0.01 mM, and above this concentration the slopes of the working curves decrease owing to the higher concentration of free dye which results in a higher absorbance and thus less enhancement of the synchronous fluorescence.

The detection limit (DT) is defined by IUPAC [26]. From the dynamic ranges and detection limits for HSA, it is very clear that this method is sensitive.

3.6. Analysis of samples

The present method was applied to determine HSA in human serum samples. Human serum samples were stored at 0–5 °C and diluted 1000-fold with deionized water just before determination. Table 2 summarized the results, which are close to the clinical data got from hospital. Therefore, the determination of HSA by this method is reliable, sensitive and practical.

Table 2
Determination results of HSA in human serum samples

Human serum sample	Content of HSA		Recovery (%) (n = 6)	R.S.D. (%)
	The clinical data (mg mL^{-1})	This method (mg mL^{-1})		
1	78.0	77.3	99.0	2.0
2	76.7	78.5	103.3	2.3
3	77.0	77.9	101.9	1.7

Table 3
Interference from foreign substances

Foreign substance	Coexisting concentration (μg mL^{-1})	Change of synchronous fluorescence intensity (%)
Arginine	8.5	−2.9
Lysine	8.0	−1.7
Citric acid	15	+3.2
Glucose	11.0	−4.8
Zn(II)	6.0	−2.1
Cu(II)	5.6	−3.6
Fe(III)	4.8	−3.9
Mg(II)	8.0	−2.6
Co(II)	10	−3.7
Al(III)	5.0	−4.8

HSA: 1 μg mL^{-1}; methyl blue: 1.0×10^{-5} mol L^{-1}; pH 4.1.

3.7. Interference test

The interference of various foreign substances was tested and shown in Table 3. The interference of citric acid and glucose is very weak. Of the tested metal ions, Co(II), Mg(II) and Zn(II) can be allowed at relatively higher concentration, but Fe(III), Al(III) and Cu(II) can be allowed only at a relatively low concentration. It can be seen from this table that this method may have its practical use.

4. Conclusion

A synchronous fluorescence spectrum has several advantages compared with the normal fluorescence spectrum, such as sensitivity, spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects. The aim of this study was to determine of HSA with methyl blue as a fluorescence probe by synchronous fluorescence method. Triphenylmethane acid dye methyl blue exists in the acidic form in the medium of pH 4.1. So, it can combine with HSA to form a complex. When HSA is added in to the dye solution, the synchronous fluorescence intensity is significantly enhanced. Then a synchronous fluorescence method to determine HSA with the advantages of simplicity, rapidity and high sensitivity and wide linear range is established based on the interaction of HSA and methyl blue. Its detection limit reached as low as 0.03 μg mL^{-1} for HSA. Its reliability is validated by detecting the HSA in human serum samples and its result is satisfactory.

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