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Fluorometric Method for Microdetermination of Human Serum Albumin Using Substituted 3*H*-Indole Compound

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A simple, sensitive and reproducible fluorometric method for determining nanoamount of protein is developed. The method is based on the reaction between protein and 3H-indole compounds in aqueous solution. Under the optimized experimental conditions, there is little interference with amino acids and most metal ions. In the detection of total amount of proteins in human serum albumin, the values obtained by this method are close to those of the BCG method. The binding reaction mechanism between this compound and human serum albumin in aqueous solution was studied using fluorescence. Their binding constant is $K_a = 8.51 \times 10^5$ L mol^{-1} and the binding site number is n = 1.2. It is confirmed that the combination is a single static quenching process. According to the resonance energy transfer theory, the distance between the molecule and tryptophan of human serum albumin is 37.3 Å and the förster energy transfer is very efficient as high as 93.7 %.

Keywords: 3*H*-Indole compound, Human serum albumin, Fluorometric method.

INTRODUCTION

Serum albumins are the most aboundant proteins in the circulatory system of a wide variety of organisms and are known to bind a variety of biological molecules, such as drug, amino acids, steroids and metals ions, in the bloodstream to their target organs[1-2]. There are many spectrophotometric methods[3-5], usually based on the ability of proteins to bind dyes, for determining the content of proteins in samples. The most frequently used assays are the Bradford, Lowry, Biuret and Bromocresol Green methods. However, these methods have some limitations. In addition to the poor selectivity of the Lowry method, the complicated procedure in the Bradford method and turbidity problems with the Bromocresol Green method, as with most spectrophotometric methods, their sensitivity is not high enough[6].

The basic information contained in fluorescence measurements relates to the molecular environment of the chromophore, fluorescence of tryptophan residues is very sensitive to the changes in their vicinity, thus it is widely due to study variations of the molecular conformations of proteins[7-8].

In this paper a new fluorometric assay of protein is described. Ethyl 2-(4-aminophenyl)-3,3-dimethyl-3*H*-indole-5-carboxylate, (Fig. 1) has sensitive fluorescence and its fluorescence can be enhanced by the addition of albumin. Based on this, the albumin is quantitatively detected. Furthermore, mechanism of the binding reaction between this compound and human serum albumin in aqueous solution was studied using fluorescence.

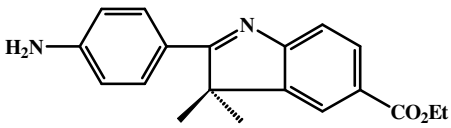

Fig. 1. Structure of substituted 3*H*-indole

EXPERIMENTAL

Absorption spectra were recorded on UV-3100 (Shimadzu) spectrophotometer using 1 cm quartz cells. Steady-state fluorescence was performed on F-4500 (Hitachi). The excitation and emission band passes were 10 nm when detecting the albumin and 5 nm when studying the binding mechanism, each solution was excited near its maximum absorption wavelength using 1 cm quartz cells. The pH values were measured with a model S-3B (Shanghai Leici).

Substituted 3*H*-indole was synthesized with reference to the original method of Popowycz[9]. The working solutions of 3*H*-indole were prepared from 2×10^{-6} mol L^{-1} in ethanol. Human serum albumin (V) was obtained from Sigma. All other chemical reagents used in this study were analytical grade. Tridistilled water was used throughout the experiments. The fresh human serum specimens (obtained from Peking University

Hospital) were diluted 1000-fold and used as working solutions for albumin detection.

Procedures: A mixture of 0.2 mL of 3*H*-indole solution, 0-2 mL of 1 mg mL^{-1} human serum albumin standard solutions or 1 mL of specimen and 2 mL 0.01 mol L^{-1} phosphate buffer (pH 7.4) was diluted to 5 mL with water. The fluorescence (λ_{ex} = 365 nm, λ_{em} = 475 nm) was measured with a 1 cm fluorometric cell at room temperature and the standard curve of fluorescence *versus* the concentrations of the human serum albumin standard solutions was plotted.

RESULTS AND DISCUSSION

The UV spectra of free 3*H*-indole system and 3*H*-indole-human serum albumin system are shown in Fig. 2a. Compared with the free 3*H*-indole system, the long-wavelength bands with peak located at about 365 nm of the conjugated 3*H*-indole system exhibit slightly blue shift, which indicates that the indole group transfers from a polar environment to a less polar environment. As can be seen from Fig. 2b, the emission is greatly enhanced when reacting with protein.

Optimum conditions for determination of protein

Optimum concentration of substituted 3*H*-indole: By fixing the concentration of human serum albumin, influences of different concentration of 3*H*-indole on fluorescence intensity was investigated. Result showed (Fig. 3) that a good linear relationship with the concentration of human serum albumin was from 2×10^{-7} to 2×10^{-6} mol L^{-1}. Due to the determination range, the 3*H*-indole concentration of 2×10^{-6} mol L^{-1} is chosen.

Effect of reaction time: At room temperature, the fluorescence intensity of mixture solution of human serum albumin and 3*H*-indole was measured per 5 min. It was found that the fluorescence intensity fell slightly.

Effect of pH: The effect of pH on protein assay was examined. The optimum pH of this assay was determined from the plot of relative fluorescence intensity (difference between 3*H*-indole-free and 3*H*-indole-HSA) against pH. As can be seen in Fig. 4, both the fluorescence of 3*H*-indole with and without human serum albumin were affected by pH. The maximum difference in the fluorescence intensity in the presence and absence of human serum albumin was obtained at pH 5-7. Due to pH of human blood, the pH to run the assay is pH 7.4.

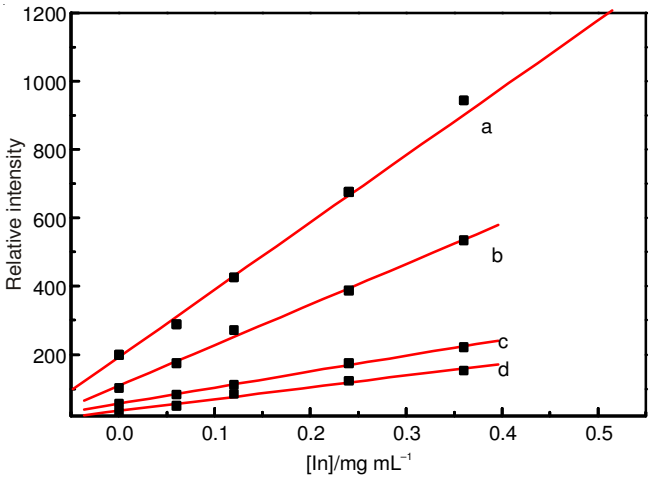

Fig. 3. Fluorescence spectra of different concentratiuon 3*H*-indole in presence of human serum albumin; (a) 2×10^{-6} mol L^{-1}; (b) 1×10^{-6} mol L^{-1}; (c) 4×10^{-7} mol L^{-1}; (d) 2×10^{-7} mol L^{-1}

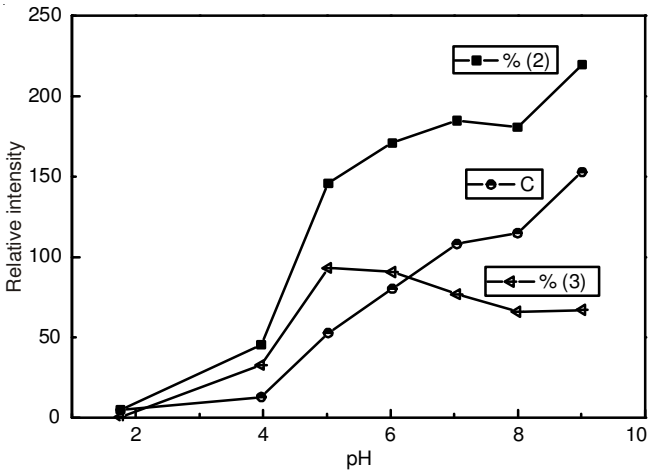

Fig. 4. Effect of pH on 3*H*-indole-human serum albumin. 1 3*H*-indole; 2 3*H*-indole + human serum albumin; 3 difference between 2 and 1. [3*H*-indole] = 2×10^{-6} mol L^{-1}; [human serum albumin] = 1×10^{-6} mol L^{-1}

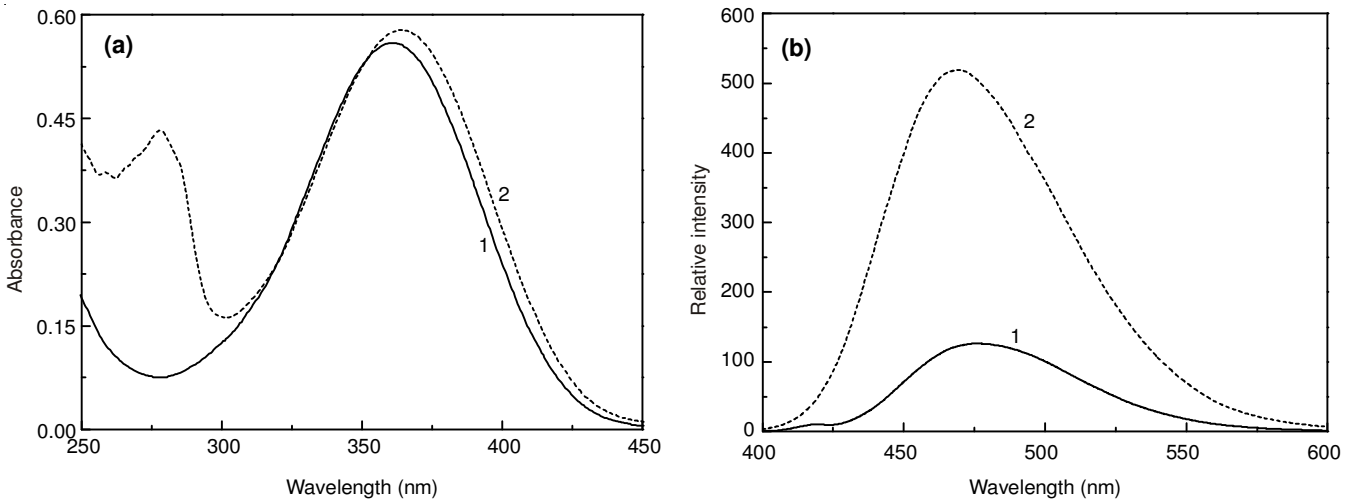

Fig. 2. Absorption; (a) [3*H*-indole] = 2×10^{-5} mol L^{-1}) and fluorescence; (b) [3*H*-indole] = 2×10^{-6} mol L^{-1}) spectra of 3*H*-indole molecule in: (1) water and (2) [HSA] = 0.3 mg mL^{-1}

Calibration graphs for different proteins

γ-G does not affect the fluorescence of 3*H*-indole (Fig. 5). So the proposed method can be used to determine albumin in serumin in serum without any preminary separation of γ-G and albumin.

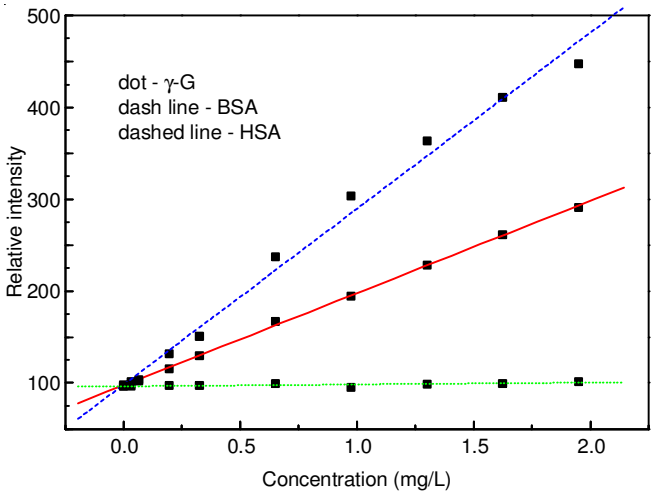

Fig. 5. Standard curves for BSA, human serum albumin and γ-G. [3*H*-indole] = 2×10^{-6} mol L^{-1}

Tolerance of foreign substances: To test the applicability of the method for the direct determination of the protein in human body fluids, the effects of several amino acid and salts were examined by adding various concentrations of the foreign substances to a fixed concentration of human serum albumin (1×10^{-6} mol L^{-1}) prior to detection. The results in Table-1 indicate that the method is free from interferences many metal ions and amino acids. These foreign substances have no obvious effect on the assay.

TABLE-1
EFFECTS OF THE FOREIGN SUBSTANCES ON THE DETERMINATION OF HUMAN SERUM ALBUMIN

No.	Foreign substance	Added/mg mL^{-1}	Relative error (%)
1	$AgNO_3$	0.2	-2.00
2	LiCl	0.2	+0.18
3	SrCl2	0.2	-0.49
4	KCl	0.2	-1.70
5	NiCl	0.2	-2.53
6	$MgSO_4$	0.2	-2.67
7	Arg	34.8	-1.42
8	Try	40.8	-0.06
9	Glu	29.2	+1.00
10	Lys	29.2	-0.37

Analytical method evaluation

Linear concentration range and limit of detection: The standard curve was constructed following the standard assay procedure and the serum samples were tested simultaneously under the same assay condition. The fluorescence intensity showed a good linear relationship with the concentration of human serum albumin was 0-400 μg. The linear regression equation was F = 95.8 + 640.8 [human serum albumin], where [human serum albumin] is in mg mL^{-1}. The correlation coefficient was 0.997. The detection limit (3σ) for human serum albumin was calculated from the standard deviation of the blank (n = 10) as 0.34 μg.

Determination of protein in blood sample: The present method was too sensitive to estimate protein in an original human serum sample. A fresh serum sample was measured after having been diluted 1000 times. Fluorescent intensity was determined at optimum assay conditions. A comparison of this method with the BCG assay is listed in Table-2. The results of three serum samples are given in Table-2.

TABLE-2
DETERMINATION RESULTS FOR HUMAN SERUM SAMPLE

	Sample 1	Sample 2	Sample 3
BCG method (mg mL^{-1})	13.6 ± 0.25	14.0 ± 0.25	20.6 ± 0.5
Fluorescence (mg mL^{-1})	18.0 ± 0.13	18.6 ± 0.13	28.6 ± 0.25
Recovery	(103.3 ± 1.3) %	(103.3 ± 3.9) %	(103.3 ± 2.7) %

Studies on binding constant of 3*H*-indole with human serum albumin: In order to study the binding constant in the system of 3*H*-indole and human serum albumin, the fluorescence quenching method was used[10-15].

According to the Stern-Volmer law, the fluorescence of a fluorophore can be quenched by aquencher with two models, dynamic quenching (or collisional quenching) and static quenching. In the appropriate concentration range of the quencher, the relationship between the quenching efficiency (F_0/F) and the quencher concentration ([ln]) satisfies the eqution:

$$F_0/F = 1 + k_{sv}\,[ln] \quad (1)$$

where F_0 and F are the fluorescence intensity of the fluorophore in the absence and presence of the quencher, respectively and k_{sv} is the Stern-Volmer quenching constant, corresponding to the slope of the plot of [ln] *vs*. F_0/F. For dynamic quenching,

$$k_{sv} = k_q\tau_0 \quad (2)$$

where k_q However, k_{sv} for static quenching decreases with increase in solution temperature as static quenching is due to the formation of a non-fluoscence ground-state complex between the fluorophore and quencher.

The intrinsic fluorescence of human serum albumin at 338.4 nm (excitation at 280 nm) is strongly by 3*H*-indole. A good linear relationship between the quenching efficiency and the concentration of 3*h*-indole in the range (0.0-4.0 × 10^{-6}) mol L^{-1} at room temperature is obtained, as shown in Fig. 6.

For the static quenching interaction, if it is presumed that there are similar and independent binding sites in the biomolecule, then the binding constant K and the number of the binding sites n for the binding of 3*H*-indole to human serum albumin can be calculated according to the following equation:

$$\log[(F_0 - F)/F] = \log K + n\log [Q] \quad (3)$$

with eqn. (2) the k and n values were calculated as 8.51×10^5 L mol^{-1} and 1.2, repectively.

Photoinduced energy transfer: The interaction between 3*H*-indole and human serum albumin in aqueous solutions has been studied with absorption and steady-state fluorescence spectra. As can be seen from Fig. 8, there is much overlap of the emission spectrum of human serum albumin and the absorption

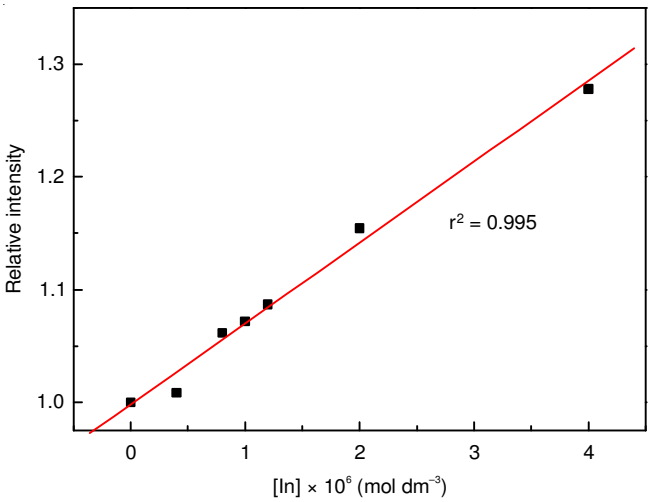

Fig. 6. Stern volmer plot of F_0/F *vs.* [ln]

spectrum of 3*H*-indole, which indicates that the photoinduced energy transfer from human serum albumin (donor) to 3*H*-indole (acceptor) is possible.

A ground state molecule bound 3*H*-indole absorbs light which been emitted from human serum albumin and converts to its first excited single state, then the rapid Förster energy transfer occurs to the nearby 3*H*-indole. According to the Förster theory[16-18], the efficiency of energy transfer is given by:

$$E = \frac{R_0^6}{R_0^6 + r^6} \quad (8)$$

where r is the distance between the donor and acceptor and is the Förster or critical transfer distance at which the energy transfer rate is equal to the decay rate. R_0 being a function of the spectral properties of a donor-acceptor pair can be represented as:

$$R_0^6 = \frac{8.8\times10^{-25}\ \kappa^2\phi_D}{n^4} \frac{\int F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d(\lambda)}{\int F_D(\lambda)\, d\,(\lambda)} \quad (9)$$

where κ^2 is the orientation factor related to the geometry of the donor-acceptor diploes and $\kappa^2 = \frac{2}{3}$ for random orientation as in fluid solution, is the refractive index of the medium, ϕ_D is the quantum yield of the donor in the absence of acceptor, $F_D(\lambda)$ is the spectral distribution of the donor emission, $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor. The value of for this human serum albumin-3*H*-indole molecule pair was found to be 37.3 Å and the In this way, the Förster energy transfer is very efficient as high as 93.7 % (Figs.7 and 8).

ACKNOWLEDGEMENTS

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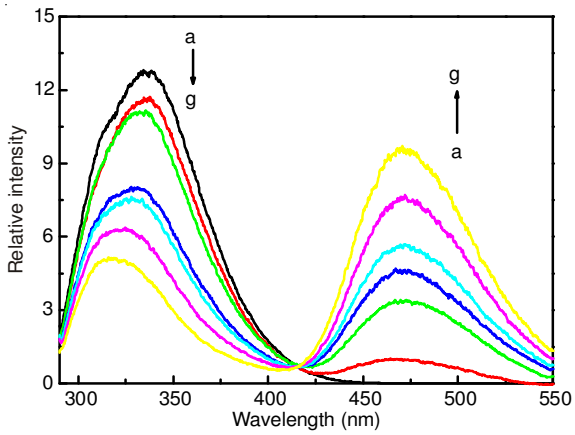

Fig. 7. Fluorescence spectra of 3*H*-indole (2×10^{-6} mol L^{-1}) in presence of HSA: 0, b:1×10^{-6} mol L^{-1}, c: 2×10^{-6} mol L^{-1}, d: 8×10^{-6} mol L^{-1}, e: 1×10^{-5} mol L^{-1}, f: 1.5×10^{-5} mol L^{-1}, g: 2×10^{-5} mol L^{-1}

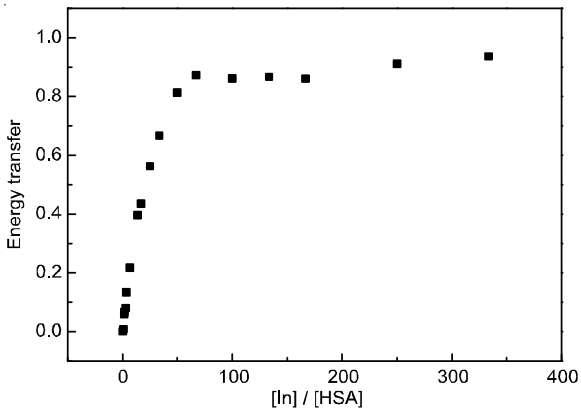

Fig. 8. Plot of [ln]/[HSA] *vs.* energy transfer efficience

REFERENCES

1. J. Flarakos, K. Morand and P. Vouros, *Anal. Chem.*, **77**, 1345 (2005).
2. Y.J. Hu, Y. Liu and X.H. Xiao, *Biomacromolecules*, **10**, 517 (2009).
3. Y.Q. Duan, H.G. Lei, S.G. Min and Z.Q. Duan, *Spectrosc. Spectr. Anal.*, **29**, 2998 (2009).
4. Y.J. Hu, Y. Liu, Z.B. Pi and S.-S. Qu, *Bioorg. Med. Chem.*, **13**, 6609 (2005).
5. P.B. Kandagal, S.S. Kalanur, D.H. Manjunatha and J. Seetharamappa, *J. Pharm. Biomed. Anal.*, **47**, 260 (2008).
6. J. Lakowicz, Principles of Fluorescence Spectroscopy, Springer, New York, edn. 3 (2006).
7. M. Mehrabi, S. Ghobadi and R. Khodarahmi, *Photochem. Photobiol. B*, **97**, 161 (2009).
8. P.B. Kandagal, S. Ashoka, J. Seetharamappa, S.M.T. Shaikh, Y. Jadegoud and O.B. Ijare, *J. Pharm. Biomed. Anal.*, **41**, 393 (2006).
9. Y.L. Chen, T.K. Xu, X.H. Shen and H. Gao, *J. Photochem. Photobiol. Chem.*, **169**, 123 (2005).
10. R. Bera, B. Sahoo, K. Ghosh and S. Dasgupta, *Int. J. Biol. Macromol.*, **42**, 14 (2008).
11. H. Du, J. Xiang, Y.A. Zhang and Y. Tang, *Bioorg. Med. Chem. Lett.*, **17**, 1701 (2007).
12. H.J. Liu, P. Li and Y.D. Zhang, *Spectrosc. Spectr. Anal.*, **29**, 1915 (2009).
13. X.M. Cao and L.M. Du, *Spectrosc. Spectr. Anal.*, **27**, 973 (2007).
14. F. Cui, L. Qin, G. Zhang, X. Yao and B. Lei, *Macromol. Biosci.*, **8**, 1079 (2008).
15. F. Cui, Q. Zhang, Y. Yan, X. Yao, G. Qu and Y. Lu, *Carbohydr. Polym.*, **73**, 464 (2008).
16. T. Förster, in ed.: O. Sinanoglu, Delocalized Excitation and Excitation Transfer, In: Modern Quantum Chemistry, Academic Press, New York, p. 93 (1965).
17. T.K. Xu, N. Li, X.H. Shen and H.C. Gao, *Spectrosc. Spectr. Anal.*, **25**, 1443 (2005).
18. T.K. Xu, X.H. Shen and H.C. Gao, *Spectrosc. Spectr. Anal.*, **25**, 857 (2005).