Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 4091–4096
Bioorganic & Medicinal Chemistry Letters

Determination of human serum albumin using an intramolecular charge transfer fluorescence probe: 4′-Dimethylamino-2,5-dihydroxychalcone

Zhicheng Xu, Weibing Yang and Chuan Dong*

Department of Chemistry, Shanxi University, Taiyuan 030006, PR China

Received 17 March 2005; revised 25 May 2005; accepted 3 June 2005
Available online 29 June 2005

Abstract—A new intramolecular charge transfer fluorescence probe, namely, 4′-dimethylamino-2,5-dihydroxychalcone (DMADHC), exhibited dramatic enhancement of fluorescence intensity with an accompanying blue shift of the emission maximum when the concentration of human serum albumin (HSA) was increased. Binding to HSA also caused a progressive shift in the absorption spectrum of DMADHC, and a clear isosbestic point appeared. The binding site number and binding constant were calculated. Thermodynamic parameters were given and possible binding site was speculated. The optimum conditions for the determination of HSA were also investigated. A new, fast, and simple spectrofluorimetric method for the determination of HSA was developed. In the detection of HSA in samples of human plasma, this method gave values close to that of the Erythrosin B method.
© 2005 Elsevier Ltd. All rights reserved.

1. Introduction

The quantitative analysis of protein continues to be a very active area because it can be used as a reference for the measurement of other components in biochemical analysis and clinical diagnoses. There are many techniques such as ultraviolet and visible absorption (UV–vis) spectroscopy,[1] fluorescence spectroscopy,[2,3] calorimetry,[4] light scattering,[5] and capillary electrophoresis[6] to detect the protein. Among them, the fluorescence method stands out since it is more widely used in peptide and protein chemistry than any other spectral detection[7]. Since the fluorescence emitted from the native protein is very weak, the emphasis of fluorescence method for the detection of protein is focusing on the probe of proteins. However, few researches were involved in using the compound with intramolecular charge transfer (ICT) behavior as a fluorescence probe for the determination of protein.

Some compounds containing both electron donor and acceptor moieties exhibit ICT fluorescence behavior whose fluorescence is sensitive to the change in the solvent properties. The emission state from ICT state has attracted great interest both in photochemistry and biochemistry.[8–10] A series of substituted chalcones, 4′-dimethylaminochalcones, show strongly intramolecular charge transfer behaviors and reasonable fluorescence quantum yields in various aprotic solvents.[11,12] A detailed study of them should therefore be of immense use in unraveling the nature of their interaction with different substances and other biological targets (e.g., membranes and protein) at the molecular level. In this letter, we report a detailed study of the interaction of substituted chalcone, namely, 4′-dimethylamino-2,5-dihydroxychalcone (DMADHC) (Fig. 1), with human serum albumin (HSA) in aqueous solutions. The binding characteristics are discussed. The performance of the

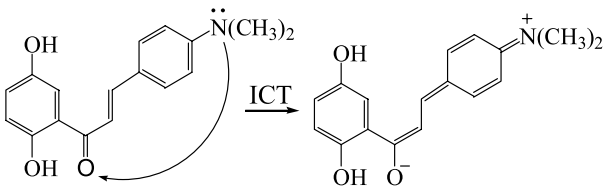

Figure 1. The molecular structure and intramolecular charge transfer process of compound 4′-dimethylamino-2,5-dihydroxychalcone (DMADHC).

Keywords: Intramolecular charge transfer; Fluorescence probe; Human serum albumin.
* Corresponding author. Tel.: +86 351 7011865; fax: +86 351 7011322; e-mail: dc@sxu.edu.cn

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.06.014

analytical system involving the interaction of DMADHC with HSA was explored and its application in determining the protein is reported.

2. Materials and methods

2.1. Reagents

The investigated chalcone DMADHC was synthesized and purified as described in the literature,[13] and testified by ^{1}HNMR and IR spectroscopy. The HSA was purchased from Biological Identification Institute of Shanghai. Phosphate buffer solution was used to control the pH of the media. All other reagents were of analytical reagent grade and were used without further purification. Double distilled water was used throughout the experiment.

2.2. Apparatus

Absorption and fluorescence spectrum were recorded on TU-1901 UV–vis spectrophotometer (PGENERAL) and F-4500 spectrofluorometer (HITACHI). Excitation and emission bandwidths were both set at 5 nm. All pH values were measured with a pHs-2 acidometer (The Second Instrument Factory of Shanghai, China). All experiments were carried out at 20 ± 1 °C.

2.3. Procedures

The concentrations of DMADHC in all the tested solutions were 10^{-5} mol L^{-1}. An appropriate amount of HSA was added. All experiments were carried out at pH 7.4 in phosphate buffer solutions except specifically indicated. All test solutions were incubated at 20 ± 1 °C for 30 min. The standard curve method was used in the quantification of trace HSA in serum samples.

3. Spectral characteristics of DMADHC in various solutions

As can be seen in Figure 2, the fluorescence spectrum suffers a strongly bathochromic shift as the solvent polarity is increased. This red shift in the emission maximum, from 487 nm in carbon tetrachloride to 533 nm in dimethyl sulfoxide, observably indicates that intensively photoinduced ICT takes place within the molecule in a singlet-excited state. The emission state of DMADHC consists of three states, the primarily less polar emission state E*, the subsequently more polar emissive state A* (corresponding to the LE state and the TICT state), and the apolar nonemissive state P*. The fluorescence of the molecule is the sum of the fluorescence emissions from states E* and A*.[11] The peak wavenumbers in the fluorescence spectrum of the compound in different solvent polarity can be correlated with the empirical Dimroth polarity parameter $E_T(30)$[14] of the solvents (refer to the inset of Fig. 2). A very good linear correlation was obtained, implying potential application of this emission parameter to probe the local microenvironment of DMADHC.

4. Spectra characteristics of DMADHC binding to HSA

The spectra characteristics associated with the binding of DMADHC to HSA can be revealed in Figure 3. Binding to HSA may cause a progressive shift in absorption spectrum of DMADHC, and its absorption maximum shifts from 413 to 422 nm. A clear isosbestic point was located at 440 nm, indicating the existence of two forms of

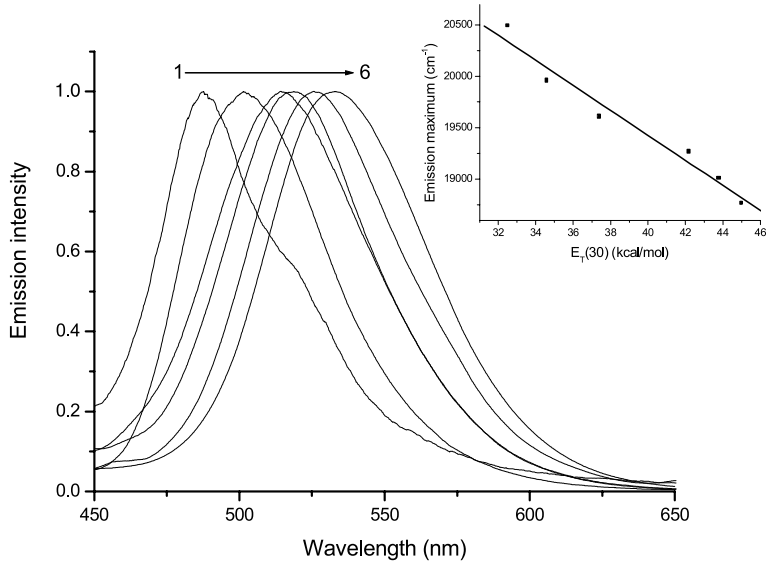

Figure 2. Normalized fluorescence spectrum of DMADHC in various solvents: (1) carbon tetrachloride, (2) diethyl ether, (3) tetrahydrofuran, (4) acetone, (5) DMF, and (6) DMSO. The arrow direction indicates increasing solvent polarity. The inset displays the linear calibration curve of The emission maximum versus the solvent polarity parameter $E_T(30)$.

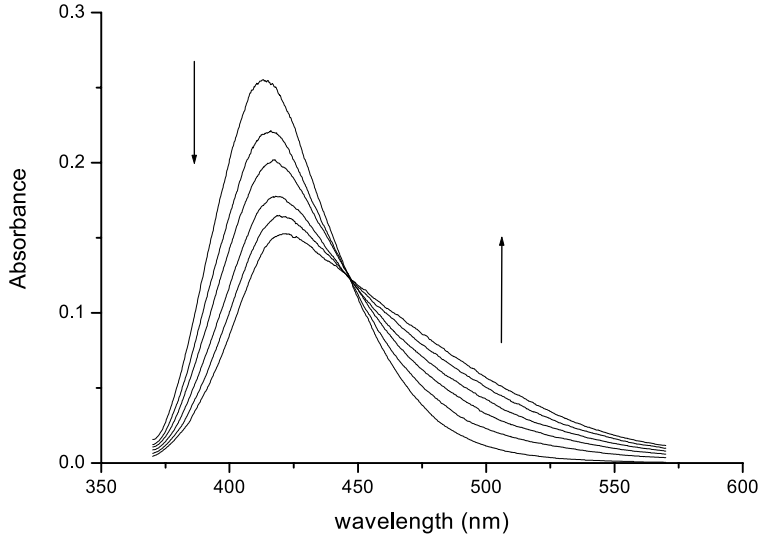

Figure 3. Absorption spectrum of 1×10^{-5} mol L^{-1} DMADHC in different concentrations of HSA at pH 7.4. HSA concentration: (1) 0, (2) 1×10^{-7} mol L^{-1}, (3) 2×10^{-7} mol L^{-1}, (4) 5×10^{-7} mol L^{-1}, (5) 1.0×10^{-6} mol L^{-1}, and (6) 2.0×10^{-6} mol L^{-1}. The arrow direction indicates increasing concentrations of HSA.

DMADHC, that is, free and bound ones. Each form of DMADHC has a unique absorption.

Figure 4 shows the ICT fluorescence emission of DMADHC in the presence of different amounts of HSA. Addition of low HSA concentration caused notable increase of DMADHC fluorescent intensity. The maximum emission wavelength is strongly blue shifted from 550 to 541, 536, 533, 531 or 530 nm, respectively. The blue shift of the emission maximum can be rationalized by the binding of DMADHC to a less polar site in HSA. The enhancement effect concluded that the DMADHC molecule has been included into the hydrophobic pocket of the host HSA whose size is consistent with DMADHC molecule where little rotation of the probe occurs during the excited state. The enhancement of the fluorescence was in proportion to the concentration of HSA and according to this a new method for determination of HSA has also been established.

5. Binding constant and binding site number

For the interaction of small molecules with macromolecules, the Scatchard plot is commonly used to characterize the binding properties in terms of measuring the binding constant and binding site number. The data for Scatchard analysis are based on the measurements of absorbance or fluorescence of interacting system,[15]

$$r/c = k(n - r), \tag{1}$$

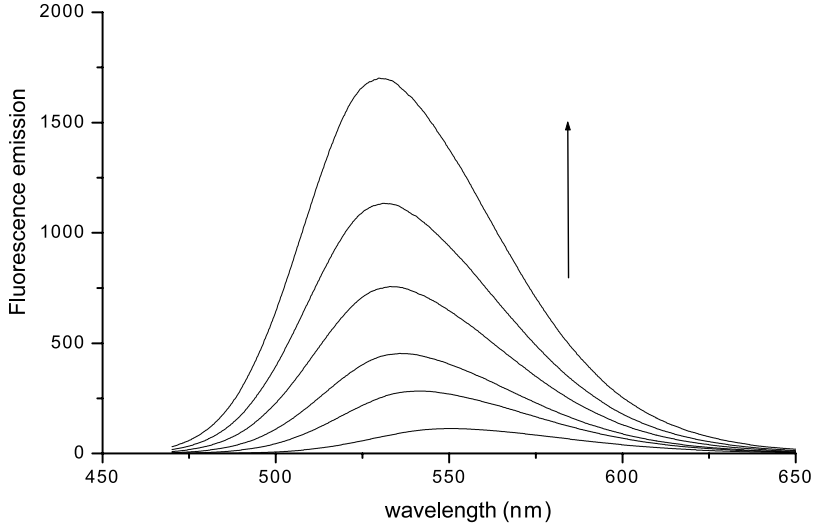

Figure 4. Fluorescence emission spectrum of 1×10^{-5} mol L^{-1} DMADHC in different concentrations of HSA at pH 7.4 HSA concentration: (1) 0, (2) 1×10^{-7} mol L^{-1}, (3) 2×10^{-7} mol L^{-1}, (4) 5×10^{-7} mol L^{-1}, (5) 1.0×10^{-6} mol L^{-1}, and (6) 2.0×10^{-6} mol L^{-1}. The arrow direction indicates increasing concentrations of HSA.

where r is the molar ratio of bound DMADHC to HSA, n denotes the binding site number, K represents the intrinsic binding constant and c is the concentration of free DMADHC. From the recorded fluorescence titration data, the binding constant and binding site number of DMADHC with HSA can be calculated. The binding constant was 1.03×10^4 L mol^{-1} and the binding site number was 0.93. These results indicate that DMADHC has intense affinity to HSA, and DMADHC–HSA complex may only have one binding site.

6. Evaluation of the interaction between DMADHC and HSA

The forces acting between a drug and a biomolecule are composed of weak interactions of molecules such as hydrogen bond formation, van der Waals forces, electrostatic forces, and the hydrophobic interaction.[16] From the thermodynamic standpoint, $\Delta H > 0$ and $\Delta S > 0$ implies a hydrophobic interaction; $\Delta H < 0$ and $\Delta S < 0$ reflects the van der Waals force or hydrogen bond formation; with $\Delta H \approx 0$ and $\Delta S > 0$ suggesting an electrostatic force.[17] Because the temperature changes of the binding interaction are minimal, the interaction enthalpy change can be regarded as a constant. Therefore, from the following equations:

$$\ln \frac{k_2}{k_1} = \left(\frac{1}{T_1} - \frac{1}{T_2}\right) \frac{\Delta H}{R}, \tag{2}$$

$$\Delta G = \Delta H - T\Delta S, \tag{3}$$

$$\Delta G = -RT \ln K, \tag{4}$$

where the values of ΔH, ΔG, and ΔS are enthalpy change, free energy change, and entropy change, respectively. ΔH and ΔS were calculated to be 5249 J mol^{-1} and 186.27 J mol^{-1} K^{-1}. It can be deduced that the interaction for the binding reaction between DMADHC and HSA is mainly a hydrophobic interaction.

7. Speculation of binding site of DMADHC and HSA

Analysis of HSA interactions within the IIA and IIIA principal ligand-binding structural domains can suggest the possible binding site for the probe molecule. Crystal structure analysis has revealed that two major ligand-binding sites, sites I and II, are located within the specialized cavities in subdomain IIA and IIIA, respectively. In particular, subdomains IIA and IIIA are delimited by a hydrophobic surface on one side and a positively charged surface on the other side, which allow them to specifically bind small aromatic carboxylic acids and negatively charged heterocyclic legends of average size, respectively.[18] Actually, a large hydrophobic cavity is present in the IIA subdomain, and the portion of the cavity in subdomain IIIA effectively eliminates hydrophobic interactions. Since the interaction for the binding reaction between DMADHC and HSA is mainly a hydrophobic interaction, we confer that DMADHC may penetrate into the hydrophobic subdomain IIA.

8. Effect of the concentration of DMADHC

The concentration of DMADHC has distinct effect on the fluorescence intensity ratio (F/F_0) for HSA existence and absence systems. Figure 5a showed the influence of the probe DMADHC concentration on the fluorescence intensities (F/F_0). With increasing concentration of DMADHC, the fluorescence intensity was gradually enhanced. But the intensity increased very slightly after the concentration of the DMADHC solutions reached 5×10^{-6} mol L^{-1}, and it dropped when the concentration reached 3×10^{-5} mol L^{-1}. This phenomenon may be due to the self-quenching behavior of DMADHC at high concentration. Thus, the final concentration of fluorescence probe DMADHC was kept at 1×10^{-5} mol L^{-1} in further experiments.

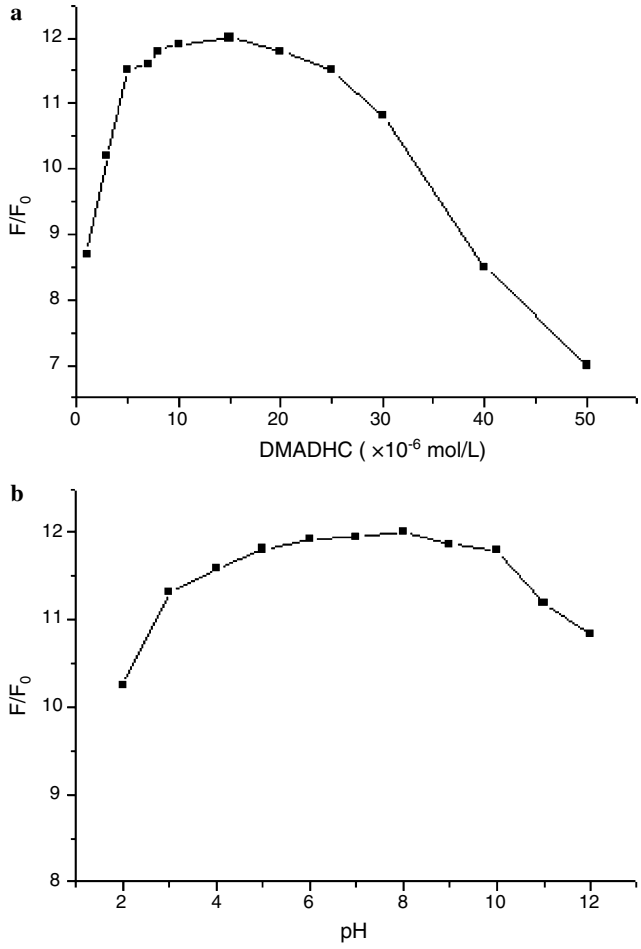

Figure 5. (a) Effect of the concentration of DMADHC. [HSA] = 1×10^{-6} mol L^{-1}; pH 7.4. (b) Effect of pH on the DMADHC standard assay. [DMADHC] = 1×10^{-5} mol L^{-1}; [HSA] = 1×10^{-6} mol L^{-1}.

9. Effect of pH

Figure 5b shows the variation of fluorescence intensity with the pH of solution. The fluorescence intensity of DMADHC solution changes only slightly when pH value varies from pH 4.0 to 10.0, but decreased out of this range. It can be inferred that the pH of solution has scarcely any effect on the emission state of DMADHC molecule except for strong acidic and alkaline environments in which HSA molecules may have been destroyed. So pH 7.4 was chosen for the subsequent experiments because it is the physiological acidity.

10. Influence of coexisting substances

Under the optimum conditions, the influence of coexisting substances such as glucose, citric acid, amino acid, and metal ions were tested. The criterion for interference was fixed at a ±10% variation of the average fluorescence intensity calculated for the established detection level of HSA.[19] The experimental results are presented in Table 1. From the results it can be seen that the coexisting substances test showed little or no influence.

11. The analytical characteristics

The linear calibration graph for determination of HSA was constructed from results obtained under the optimal condition. The linear calibration equation, precision, and limit of the detection were obtained according to the general procedure. The fluorescence system reported here was used to determine HSA ranging from 1 to 95 μg ml^{-1} with a detection limit (3σ) of 0.5 μg ml^{-1}. The correlation coefficient is 0.99. This method exhibits good reproducibility, with a relative standard deviation of 1.15% obtained from six separate determinations for 50 μg ml^{-1} HSA.

12. Determination of HSA in samples of human plasma

The proposed method was employed to determine HSA in samples of human plasma. The samples of human plasma, obtained from the hospital of Shanxi University, were stored below −5 °C and diluted appropriately to be within the linear range of determination of HSA. A portion (1.0 ml) of this sample solution was analyzed, using the standard calibration method. Comparison of this method with the EB assay[20] is listed in Table 2.

Table 1. Influence of coexisting substances

Coexisting substance	Concentration ($\times 10^{-6}$ mol L^{-1})	ΔF%
Glucose	2.02	−1.53
Citric acid	8.40	−3.72
Tryptophan	4.72	+5.89
Leucine	6.84	+4.46
Valine	6.12	+1.25
Cysteine	3.48	+2.01
Methionine	3.22	+4.53

[HSA] = 1×10^{-6} mol L^{-1}; [DMADHC] = 1×10^{-5} mol L^{-1}; pH 7.4.

Table 2. Determination of HSA in human plasma samples

Sample No.	Present assay—found protein (μg/ml) ($n = 5$)	EB assay[a]—found protein (μg/ml) ($n = 5$)	RSD (%)	Recovery (%)
1	69.3	67.8	1.6	98.8
2	67.5	67.4	1.3	95.6
3	71.6	69.7	1.9	103.0

[a] Ref. 20.

As can be seen in Table 2, the results of these serum samples in the present method are close to those obtained by the EB assay. The recoveries of these samples are 95.6–103.0%, demonstrating that the present method offers an excellent, accurate, and precise method for the determination of HSA in human plasma.

In conclusion, the fluorescence probe of DMADHC, with intramolecular charge transfer characteristics, is sensitive to microenvironment property. It is found that new ICT-based fluorescent probe DMADHC could interact with HSA, which resulted in the significant enhancement of DMADHC fluorescent intensity. Based on this, a means of sensitive determination of protein is established. Detecting the protein in real sample validates its reliability and its result is satisfactory. Therefore, this method is potential to apply to biochemistry and clinical practice. Further work is necessary for a more basic understanding of the mechanism of their binding action.

Acknowledgment

This work is supported by the National Natural Science Foundation of China (No. 20275022). All the authors express their deep thanks.

References and notes

1. Kosman, D. J.; Hassett, R.; Yuan, D. S.; Mc, C. J. *J. Am. Chem. Soc.* **1998**, *120*, 4037.
2. Sun, C. X.; Yang, J. H.; Wu, X.; Su, B. Y.; Liu, S. F. *Chem. Phys. Lett.* **2004**, *398*, 343.
3. Wang, L.; Wang, L. Y.; Zhu, C. Q.; Wei, X. W.; Kan, X. W. *Anal. Chim. Acta* **2002**, *468*, 35.
4. Takano, K.; Yamagata, Y.; Fuiii, S.; Yutan, K. *Biochemistry* **1997**, *36*, 688.
5. Yao, G.; Li, K. A.; Tong, S. Y. *Anal. Lett.* **1998**, *31*, 1689.
6. Lee, I. H.; Pinto, D.; Arriaga, E. A.; Zhang, Z.; Dovichi, N. *J. Anal. Chem.* **1998**, *70*, 4546.
7. Lee, J. K.; Ross, R. T. *J. Phys. Chem. B* **1998**, *102*, 4612.
8. Wang, S. L.; Ho, T. I. *J. Photochem. Photobiol. A: Chem.* **2000**, *135*, 119.
9. Fayed, T. A. *Colloids Surf., A* **2004**, *236*, 171.
10. Zhang, X.; Guo, L.; Wu, F. Y.; Jiang, Y. B. *Org. Lett.* **2003**, *5*, 2667.
11. Jiang, Y. B.; Wang, X. J.; Lin, L. *J. Phys. Chem.* **1994**, *98*, 12367.
12. Wang, P. F.; Wu, S. K. *J. Photochem. Photobiol. A: Chem.* **1995**, *86*, 109.
13. Li, H. P.; Xie, H. Z.; Wang, P. F.; Wu, S. K. *New J. Chem.* **2000**, *24*, 105.

14. Suppan, P. *J. Photochem. Photobiol. A: Chem.* **1990**, *50*, 293.
15. Huang, C. Z.; Li, Y. F.; Liu, X. D. *Anal. Chim. Acta* **1998**, *375*, 89.
16. Nie, L. H.; Zhao, H. C.; Wang, X. B.; Wang, X. *J. Beijing Normal Univ. (Nat. Sci.)* **2001**, *37*, 87.
17. Ross, D. P.; Subramanian, S. *Biochemistry* **1981**, *20*, 3096.
18. Dufour, C.; Dangles, O. *Biochim. Biophys. Acta* **2005**, *1721*, 164.
19. Liu, W.; Huang, C. Z.; Li, Y. F. *Analyst* **2002**, *127*, 1392.
20. Ma, C. Q.; Li, K. A.; Tong, S. Y. *Anal. Chim. Acta* **1996**, *333*, 83.