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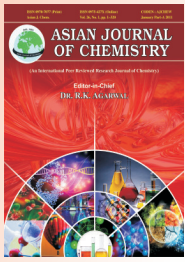

Interaction Between Phenanthorline and Proteins: A Fluorescence Spectroscopy-Based Study

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Serum albumin with cardinal physiological functions is the most abundant protein in blood plasma. The concentration of serum albumin is an index of physical health and disease. Spectrophotometry was always used to determinate the concentration of serum albumin. In present study, a novel method for the determination of proteins was established based on the enhanced fluorescence intensity derived from the binding interaction of phenanthorline with proteins in the CH_3COOH-CH_3COONa buffer at pH 5.98. Underlying the excitation wavelength at 270 nm and the emission wavelength at 366 nm, the enhancement of fluorescence intensity was proportional to the concentration of proteins. The linear range for the calibration graph of human serum albumin was 20-160 μg/mL and the detection limit was 24.12 μg/mL. The recovery was 95.0-105.3 %. The method was sensitive, accurate, required fewer samples and was tolerant of many foreign substances.

Keywords: Phenanthorline, Human serum albumin, Fluorometry.

INTRODUCTION

Protein determination is important in clinical medicine and the life sciences. Serum albumin with cardinal physiological functions is the most abundant protein in blood plasma. The concentration of serum albumin is an index of physical health and disease. Spectrophotometry was always used to determinate the concentration of human serum albumin. However, fluorescence spectroscopy has been applied to analyze biomacromolecules because of the requirement for fewer samples, ease of operation and high sensitivity[1-4]. However, fewer reports showed uses of fluorescence spectroscopy for determination of human serum albumin.

Phenanthorline is mainly used for the determination of inorganic metal ions[5-7]. The interaction between phenanthorline and proteins has not been reported. Here, the interaction between phenanthorline and proteins was studied and a novel quantitative determination method for proteins developed. Due to differences in fluorescence intensity between phenanthorline and phenanthorline-protein complexes, trace amounts of protein can be determined with good reproducibility and high sensitivity. This method was better than a Coomassie Brilliant Blue assay and easy to do. The effects of ion strength on the staining reaction were also studied. The amount of total proteins in human serum samples was determined satisfactorily.

EXPERIMENTAL

An F-4500 fluorescence spectrophotometer (HITACHAI, Japan) was used for recording the fluorescence spectra and a PHS-3C Accurate pH meter (Shanghai Leici Instruments Company, China) was used to measure pH values.

Proteins: Human serum albumin (HSA), bovine serum albumin (BSA), lysozyme (Lys), pepsin (Pep) and casein were purchased from Sigma. The stock protein solutions were 1 mg/mL.

Analytical-reagent grade phenanthorline was obtained from Shanghai Chemical Plant (China) and a 1.00×10^{-4} mol/L aqueous solution was used as the working solution. A CH_3COOH-CH_3COONa buffer solution was used to control pH values of the tested solutions. All other reagents were analytical or guaranteed reagent grade. Doubly-deionized water was used throughout.

Procedure: In most experiments, 1.2 mL of phenanthorline (1.00×10^{-4} mol/L), 2 mL of CH_3COOH-CH_3COONa buffer solution (pH 5.98) and certain amount of protein standard solutions or samples were added to 10 mL volumetric flasks, then diluted to the mark and mixed thoroughly. Fluorescence spectra were recorded at 5 nm or fluorescence intensities were measured at λ(ex) 270 nm and λ(em) 366 nm with a 1 cm cell.

RESULTS AND DISCUSSION

Fluoresence spectra and reaction mechanism: The fluorescence spectra of phenanthorline-human serum albumin complex are shown in Fig. 1. The maximum excitation wavelength of phenanthorline was 270 nm and its emission wavelength was 366 nm in the CH_3COOH-CH_3COONa buffer at pH 5.98. The fluorescence intensity of the reaction mixture was enhanced after human serum albumin treatment of the phenanthorline. The fluorescence intensity of the CH_3COOH-CH_3COONa buffer had no effect on the system.

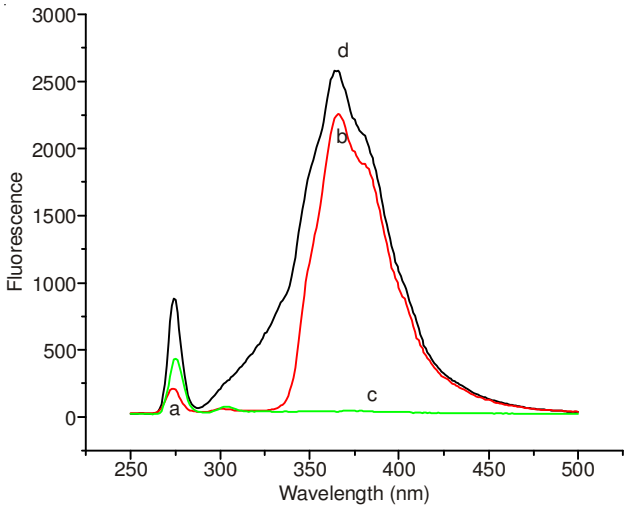

Fig. 1. Fluorescence spectra (pH 5.98). a.b. CH_3COOH-CH_3COONa + phenanthroline; c. CH_3COOH-CH_3COONa; d. CH_3COOH-CH_3COONa +phenanthorline + human serum albumin $C_R = 1.2 \times 10^{-5}$mol/L $C_{HSA} = 1.47 \times 10^{-6}$ mol/L

In acidic medium at pH 5.98, phenanthorline had a negative charge and the proteins had positive charges. Therefore phenanthorline reacted with the proteins to form a stable compound (phenanthorline-human serum albumin) by electrostatic attraction. The enhancement of fluorescence intensity was proportional to the concentration of proteins.

Effect of pH and buffer agents: The fluorescence intensity of phenanthorline-human serum albumin was affected by pH. Some buffer systems were tested (such as B-R) and results indicated that the CH_3COOH-CH_3COONa buffer was better than others; 2.0 mL of CH_3COOH-CH_3COONa buffer (pH 5.98) was chosen to control the pH of the solutions. At pH 5.98, the fluorescence intensity reached a maximum and the complexes were at their most stable.

Effect of phenanthorline concentration: The phenanthorline was tested for 1.47×10^{-6} mol/L human serum albumin. The enhancement of fluorescence intensity of the complex was proportional to the concentration of phenanthorline and the fluorescence intensity of system was unchanged at 1.0-1.8 mL ($C_R = 1.0\text{-}1.8 \times 10^{-5}$ mol/L) of phenanthorline. A 1.2 mL (final concentration of phenanthorline of 1.2×10^{-5} mol/L) was taken for the assay.

Effect of reaction time and temperature: The effect of reaction time was tested at 15 ºC (room temperature), 30 ºC and 35 ºC (water bath), respectively. The interaction between phenanthorline and human serum albumin occurred rapidly at the aforementioned temperatures and its sensitivity remained unchanged. We choose room temperature conditions for convenience and stability: the complex was stable for at least 100 min.

Effect of ion strength: The effect of ion strength on the reaction of phenanthorline with human serum albumin was tested with NaCl. When the concentration of NaCl reached 0.003 mol/L, the fluorescence intensity of the system was increased by about 6.25 %. When the concentration of NaCl reached 0.014 mol/L, the fluorescence intensity decreased by 10 %. The results indicated that the human serum albumin had mainly combined with phenanthorline by electrostatic attraction.

Effect of surfactants: Several types of surfactants were introduced to the phenanthorline-human serum albumin reaction system. They were: Neutral Triton X-100, OP, cationic surfactant CPB and anionic surfactant SDS, which quenched the fluorescence. When the concentrations of CPB and SDS reached 0.001 %, the fluorescence intensity decreased by 21.58 and 11.25 %, respectively. When the concentration of OP reached 0.005 %, the fluorescence intensity decreased by 15.45 %. When the concentration of Tritton X-100 reached 0.025 %, the fluorescence intensity was quenched completely.

Precision and detection limit of the assay: Ten blank solutions were determined. The standard deviation of the method was 37.6965 and the detection limit (3s/K) was 24.12 μg/mL. Ten standard human serum albumin solutions were measured, where each was kept at 1 mg human serum albumin, the average fluorescence intensity of system was 2809.9, RSD was 0.68 %[8].

Calibration curves: Calibration curves, linear range, correlation coefficients (r) and detection limit of bovine serum albumin, human serum albumin, casein, pepsin (Pep) and Lys were obtained under the aforementioned conditions All analytical parameters are listed in Table-1. Their sensitivities exhibited some differences because of the different numbers of amino group in different proteins; in addition, the binding method by which amino groups attached to the proteins were not identical. No spectrophotometric method could currently have overcome this problem.

Effect of coexisting substances: The influence of coexisting substances, such as metal ions and anions, was tested under the conditions given above. The concentration of human serum albumin was 0.1 mg/mL. Results obtained are presented in

TABLE-1
CALIBRATION CURVE FOR PROTEINS

Protein	Linear equations	Linear range (mg/mL)	r	Detection limit (μg/mL)
BSA	F = 3.471C + 2387.57	20-160	0.9930	32.58
HSA	F = 4.688C + 2007.83	20-160	0.9984	24.12
Casein	F = 5.641C + 2133.18	20-160	0.9908	20.05
Pep	F = 5.203C + 1726.11	20-160	0.9906	21.74
Lys	F = 2.997C + 2247.22	20-160	0.9904	37.74

Table-2. Experimental results indicated that none of them exerted significant interference on the assay. The fluorescence intensity of the system was enhanced after Zn^{2+} was added to the system. EDTA (1 %, 0.3 mL) was added to the system to mask the Zn^{2+} (0.01 mg). The fluorescence of the system was quenched after Cu^{2+} was added to the system. $Na_2S_2O_3$ (1 %, 0.5 mL) was added to the system to mask the Cu^{2+} (0.5 µg).

TABLE-2
EFFECTS OF FOREIGN SUBSTANCES ON THE DETERMINATION OF 0.1 mg/mL HUMAN SERUM ALBUMIN

Substances	Added (mg/L)	Error (%)	Substances	Added (mg/L)	Error (%)
K^+	7.0	0.48	Fe^{3+}	0.004	-2.1
Na^+	7.0	0.48	NO_3^-	48.6	1.56
Ca^{2+}	3.0	0.83	Cl^-	0.011	0.53
Mg^{2+}	1.8	-0.83	SO_4^{2-}	0.09	1.52
Mn^{2+}	0.05	0.37	Ac^-	0.024	-0.49

There is minimal metal ion content in the serum samples[9]. Samples have to be diluted 100 times in the determination and do not ordinarily require masking agent treatment.

Application to human serum assay: Based on this method, which had a feature of similar response to many proteins, the concentration of total proteins of human serum samples was directly determined. Results are presented in Table-3. Compared with a Coomassie Brilliant Blue assay[10], this method had good recovery and reproducibility. There were disadvantages to using a Coomassie Brilliant Blue assay such as: the difficulty in reagent preparation, the instability of the reagents, surface adsorption of reagents and the poorer reproducibility. This proposed method will be able to avoid above disadvantages of the Coomassie Brilliant Blue assay.

TABLE-3
ASSAY RESULTS OF PROTEINS OF SAMPLE (n = 5)

Method	Sample	Protein (mg/mL)	RSD (%)	Added (mg)	Recovery (%)
Phenanthorline	1	76.01	0.48	0.5	103.2
	2	64.32	0.79	0.5	105.3
	3	61.45	1.03	0.5	95.0
CBB G-250	1	80.50	2.56	0.04	110.4
	2	66.72	2.78	–	–
	3	60.35	3.01	–	–

Conclusion

A novel method is successfully established for the determination of proteins by phenanthorline. Trace amounts of protein can be determined with good reproducibility and high sensitivity. The method is also better than a Coomassie Brilliant Blue assay and easy to perform.

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