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Studies on the Phenylfluorone-Mo(VI) Complex as Interacting Mode Spectroscopic Probe of Protein in OP Microemulsion Medium

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Abstract: The application of phenylfluorone (PF)-Mo(VI) complex as a spectroscopic probe is studied. In the presence of OP microemulsion at pH 3.04, PF-Mo(VI) complex combines protein rapidly to form a stable compound and the absorbance at 527 nm is in proportion to the concentration of protein in the range 0~16 μg mL^{-1} for bovine serum albumin (BSA). OP microemuslion media is introduced into protein determination, it has increased markedly the sensitivity of the system. The molar absorption coefficient was 5.98×10^6 L mol^{-1} cm^{-1} for BSA. The assay, with sensitivity, simplicity and tolerance to many foreign substances, is applied to the determination of protein in samples with satisfactory results. Moreover, the binding number of BSA with the complex, which is determined by molar ratio and slope ratio methods, is in good agreement.

Keywords: Protein, spectrophotometry, PF–Mo(VI) complex, microemulsion, enhanced sensitivity.

As we know, proteins are the elements of life. The qualitative and quantitative analysis of proteins is becoming more and more important. Especially in the quantitation, because natural fluorescence intensity of proteins is very weak in the visible region, usually some spectroscopic probes have been employed for the investigation. Such probes as metal and dye are commonly used for determination of protein by spectrophotometry1,2. However, the methods mentioned above have some limitations. Dye-metal spectrophotometric method has received much attention in the determination of protein at trace levels because it has high sensitivity, long stability and good selectivity. Sulfochlorophenol-Cu(II)3, chromeazurol S-Al(III)4 and so on have been put forward. Until now no studies on the interaction between protein and the phenylfluorone-Mo(VI) complex have been reported. In this paper, OP microemuslion, used as the media, is introduced into protein determination. The sensitivity of the system has been increased, due to its sensitization and solubilization. The effect of PF–Mo(VI) with BSA in microemulsion is different. The detailed description of procedures is presented below.

Microemulison, which consists of surfactant, cosurfactant, water and oil at appropriate ratios, is colorless, transparent or subtransparent, low viscosity and thermodynamically stable system. Compared with micellar system, the microemulsion has lower surface tension and strong soluble power to organic and inorganic substances.

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Microemulsion, as the media, is not only applied to the determination of many metal ions by different methods [5,6] in our previous research, but also firstly introduced to protein determination. It is a higher sensitive method for micro determination of proteins. The precision and the accuracy of the proposed method are satisfactory.

Experimental

A Shimadzu Model UV-3000 Spectrophotometric with 10 mm quartz cells was employed for absorption spectral records and absorbance measurements. A PHS-3B acidimeter was used in determining pH values.

The protein concentrations were 100 μg mL^{-1}. OP microemulsion was prepared with OP : *n*-butanol : *n*-heptane : water =5.0 : 3.3 : 0.8 : 90.9 (mass ratio). PF solution (3.0×10^{-4} mol L^{-1}) was prepared by dissolving 0.0093 g of PF in ethanol containing several drops of 1: 1 sulfuric acid and diluted to 100 mL in a volumetric flask with pure ethanol. The Mo(VI) working solutions were made by suitable dilution of stock solution as required. A pH 3.04 Clark-Lubs buffer was used to control the acidity of tested solution. Unless otherwise mentioned, all chemicals were of analytical grade and doubly distilled water was used throughout this experiment.

To a 10 mL color comparison tube, solutions were added in the following order: 1.5 mL OP microemulsion, 2.0 mL pH 3.04 buffer solution, 1.0 mL of 3.0×10^{-4} mol L^{-1} Mo(VI), 1.2 mL of 3.0×10^{-4} mol L^{-1} PF and definite standard proteins. Then the mixture was diluted to 10 mL with doubly distilled water and kept static for 15 min. Finally, the absorbance of the solution was measured at 527 nm with 10 mm cell against the reagent blank.

Results and Discussion

Under optimum experimental conditions, the spectra of PF, PF-BSA, PF-Mo(VI)-BSA, and PF-Mo(VI) were recorded Little difference of absorption spectra between PF-BSA and PF solution in the absence of Mo(VI) was observed since BSA did not react with PF. But the absorption spectra changed a lot when Mo(VI) was added to PF-BSA system. It caused a red shift of the absorption maximum by about 37 nm. What is more, the absorbance at 527 nm decreased in proportion to the amount of BSA. So PF-Mo(VI) complex can serve as a spec- troscopic probe.

In order to enhance the sensibility of developing system, OP, PVA 124, Tween 20, Tween 80 microemulsion and each corresponding micellar solution were selected for investi- gating the effect of medium on ΔA. The results showed that each microemulsion was more sensitive than its corresponding micelle. For microemulsion media, their sensitivities were as follows:

OP > Triton X-100 > Tween 20 > Tween 80 > PVA 124

Moreover, 1.5 mL OP microemulsion was the preferred additive.

The effect of solution acidity was studied and the results showed that the absorbance was nearly constant when pH was in the range of 2.60 ~ 3.20. Therefore, pH 3.04 buffer solution was selected for further studies.

The ratio of PF to Mo(VI) was also examined. When the ratio is between 1 : 1 and

1.4 : 1, ΔA reached a maximum value and kept constant. The ratio more than 1.4 : 1or less than 1 : 1 led to the decrease of ΔA. Accordingly, the experiment was carried out with 3.6×10^{-5} mol L^{-1} PF and 3.0×10^{-5} mol L^{-1} Mo(VI) in the final volume of 10 mL.

The formation time is 5 min and the system was stable for 2 h at room temperature. After that the absorbance decreased gradually even the precipitate appeared.

At pH 3.04, most proteins present as cationic forms since their isoelectric points are all above 3.04, and PF-Mo(VI) complex exists as anionic forms, which means that proteins can use the protonated amine groups of amino acid residue in the protein to bind with PF-Mo(VI) complex by an electrostatic force. When the ionic strength was higher than 2 % (NaCl), the absorbance of the solution was dropped. This phenomenon was ascribed to the decrease of interaction between BSA and PF-Mo(VI) complex because the electrostatic shielding effect of charges on BSA was strengthened with the increasing ionic strength.

The absorbance dropped in the presence of organic solvents such as methanol, ethanol and acetone due to the change in micro environment for the binding reaction. So the con- centration of ethanol should be controlled to a low level. After BSA was heated in a boiling water bath for 30 min, ΔA fell approximately 14.3 %, suggesting that the secondary and tertiary structures of BSA were destroyed.

The influence of various ions, amino acids and glucose was examined at 4 μg mL^{-1} BSA under the conditions of the general procedure. The results indicated that most substances do not interfere with the determination of BSA in the proposed method.

Calibration curves for BSA, HSA, Ova and Lys were constructed at pH 3.04 according to the standard procedure. All the results of analytical parameters were presented in **Table 1**.

The spectrophotometry compared with other reported method was listed in **Table 2**. The method was more sensitive than dye-metal complex spectrophotometric assays, including those using other complexes of trihydroxyfluorone reagents.

To the some extent, the binding number can reflect on the binding ability and the sensitivity. Molar ratio method and slope ratio method were employed to measure the binding number of PF-Mo(VI) complex and BSA. The result was very close to each other and the value was 96.

Table 1 Analytical parameters for proteins

Proteins	Linear regression equation (ρ: μg mL^{-1})	Linear range (μg mL^{-1})	r	ε ($Lmol^{-1}cm^{-1}$)
BSA	A =0.0485+0.0758ρ	0~16	0.983	5.98×10^{6}
HSA	A =-0.0232+0.0801ρ	0~16	0.993	5.05×10^{6}
Ova	A =-0.0419+0.0371ρ	0~16	0.995	1.14×10^{6}
Lys	A=0.0889+0.0336ρ	0~16	0.991	7.98×10^{5}

Table 2 Comparison of spectrophotometric methods for protein determination

Method	Determination object	Linear range ($\mu g\ mL^{-1}$)	ε ($Lmol^{-1}cm^{-1}$)
Sulfochlorophenol-Cu(II)[3]	BSA	0~160	4.19×10^5
Chromeazurol S-Al(III)[4]	BSA	1.25~25	1.20×10^6
4,5-Dibromophenylfluorone-Ti(IV)[7]	BSA	0~6	2.72×10^6
Dibromohydroxyphenylfluorone-Mo(VI)[8]	BSA		8.27×10^5
4-azochromotropic acid phenylfluorone-Mo(VI)[9]	BSA	0~18	1.28×10^6

Sample Determination

Prior to the determination, human serum samples were diluted 1000-fold with double distilled water. But fresh human urine did not need to pretreatment. A suitable amount of the sample solution was transferred to a 10 mL color comparison tube and determined by the recommend procedure. The recovery of protein was in the range of 96.5 % and 106 %. The relative standard deviations were in all instances less than 3.1 %. All these results presented sufficient precision and high accuracy.

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