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SHORT COMMUNICATION

DETERMINATION OF SERUM ALBUMIN WITH TRIBROMOARSENAZO BY SPECTROPHOTOMETRY

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ABSTRACT. The reaction of tribromoarsenazo(TB-ASA) with serum albumin in the presence of emulgent OP was studied by spectrophotometry. In a Britton-Robinson buffer solution at pH 2.9, tribromoarsenazo and bovine serum albumin can immediately form a red compound in the presence of emulgent OP with a maximum absorption wavelength at 354 nm. The presence of emulgent OP can increase the reaction sensitivity and the compound stability. The molar absorptivity of the compound is $\varepsilon_{354\ nm} = 6.13 \times 10^5\ M^{-1}\cdot cm^{-1}$. Beer's law is obeyed over the range of 5.0-75.0 $mg\cdot L^{-1}$ for bovine serum albumin. The present method was applied to the determination of the total proteins in human serums with satisfactory results.

KEY WORDS: Serum albumin, Tribromoarsenazo, Emulgent OP, Human serums, Spectrophotometry

INTRODUCTION

Protein is a necessary nourishment component for human body and has many aspect functions. It is closely related to cell structure, enzyme, hormone, virus, immunization, genetics, the origin and evolution of life, etc. In biochemistry and clinical analysis, protein is a routine test item. Therefore, the quantitative determination of protein is of great interest. In the determination of protein, photometric method for protein has the advantage of cheap instrument and easy operation and it is still investigated until now. Some dyes that mainly include anion ones such as Coomassie Brilliant Blue [1], bromophenol blue [2], methyl orange [3], eosine B [4], bromocresol green [5], and bromophenol blue [6] have been used in the determination of protein. However, these methods have their characteristics as well as drawbacks. For instance, for the Bradford method, there is poor sensitivity, bad anti-interference, poor linearity between the absorbance of the Coomassie Brilliant Blue G-250 dye-protein complex and the concentration of protein, and inconvenience in operation. Tribromoarsenazo (TB-ASA), 3-[(2,4,6-tribromophenylazo)]-6-(2-arsenophenylazo)-4,5-dihydroxynaphthalene-2,7-disulfonic acid], is an unsymmetric bis-arylazo derivative of chromotropic acid, which was used as a chromogenic reagent of rare earths [7]. The present study discovered that in a weak acid medium TB-ASA can react with bovine serum albumin (BSA) to form a red compound. The presence of emulgent OP can enhance the stability of the system. The sensitivity of the colored reaction is very high, $\varepsilon_{354\ nm} = 6.13 \times 10^5\ L\cdot mol^{-1}\cdot cm^{-1}$. The stability of the system is over 9 h. It is highly selective. The method is better than the classical Coomassie Brilliant Blue. It has been applied to the determination of total proteins in human serum samples with satisfactory results. The method is suitable for the routine analysis of protein.

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EXPERIMENTAL

Reagent and apparatus

Bovine serum albumin (BSA) working solution (500 mg·L^{-1}) was prepared by dissolving 0.0500 g of BSA (Beijing Aoboxing Biotechnology Co. Ltd., China, Molecular weight 65000) in 100 mL of doubly deionized water and stored at about 4 ^{o}C. The aqueous tirbromoarsenazo (TB-ASA, $C_{22}H_{14}AsBr_3N_4O_{11}S_2{\cdot}4H_2O$, Shanghai Changke Research Institute for Reagent, China) solution (5.0 x 10^{-4} M) was prepared by dissolving 0.0480 g of TB-ASA in 100 mL of doubly deionized water. A 0.5 % (w/v) OP emulgent (The Fifth Reagent Plant of Shengyang, China) solution was prepared by dissolving 0.500 g of OP in 100 mL of water. A pH 2.9 Britton-Robinson (B-R) buffer solution was obtained by mixing 17.5 mL of 0.2 M^{-1} sodium hydroxide with 100 mL of mixed-acid solution containing phosphoric acid, acetic acid, and boric acid each with a concentration of 0.04 M. Absolute ethanol (Beijing Beihua Fine Chemicals Co. Ltd., China) was used. All other reagents and materials used were of analytical reagent grade or the best grade commercially available unless otherwise stated. Doubly deionized water was used throughout.

All the absorption spectra and absorbance measurements were carried out on a 722S spectrophotometer (Shanghai Lingguang Technology Co., China) equipped with 10 mm cells. A Model pHS-3C acidimeter (Shanghai Fine Scientific Instrument Co., China) was used for the pH measurements.

Analytical procedure

Into a 10 mL calibrated flask were successively placed 2.0 mL of 0.50 % (w/v) emulgent OP solution, 2.0 mL of B-R buffer solution of pH 2.9, 1.0 mL of 5.0 x 10^{-4} M TB-ASA solution, 2.0 mL of absolute ethanol, 1.0 mL of 500 mg·L^{-1} BSA solution. The mixture was diluted to the mark with water and mixed well. The absorbance (A_1) of the solution at 354 nm was measured against water. The absorbance (A_2) of the blank sample without protein was obtained under the same conditions. Then, the absorbance difference ($\Delta A = A_1 - A_2$) was calculated. The protein content was obtained by the calculation of the linear regression equation of calibration curve between ΔA and the amount of protein.

RESULTS AND DISCUSSION

Absorption spectra

The absorption spectra of the TB-ASA, (TB-ASA)-protein systems in the presence of emulgent OP-100 at pH 2.9 are shown in Figure 1. The absorption maxima of the two systems are both at 524 nm. The absorbance of the colored system increases after BSA is added. This indicates that a compound between BSA and TB-ASA formed. However, the absorbance maximum difference (curves a, b) is at 354 nm. At this wavelength the sensitivity is maximum. Based on this characteristic, the measurement wavelength was selected to be 354 nm.

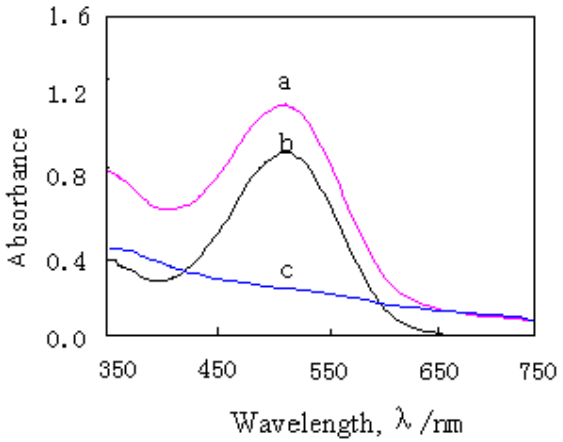

Figure 1. Absorption spectra of (a) (TB-ASA)-BSA compound (against water), (b) reagent blank (against water), and (c) (TB-ASA)-BSA (against reagent blank).

Effect of surfactant

The effect of four surfactants, neutral surfactant Tween-80, emulgent OP, cation surfactant cetyltrimethylammonium bromide (CTMAB), and anion surfactant sodium dodecyl sulfate (SDS) on the system was investigated. When no surfactant was added, a colored complex was immediately precipitated. After Tween-80, OP, CTMAB or SDS was added, the hyperchromic reactions took place and the stability and sensitivity of the system increased. The addition of CTMAB or SDS resulted a little increase in the sensitivity and stability of the system while the addition of emulgent OP resulted in better sensitivity and stability of the system. The maximum and constant absorbance was observed upon addition of 1.5-2.2 mL of 0.50 % (w/v) OP solution in a final volume of 10 mL. 2.0 mL was appropriate. The absorbance and stability of each system are listed in Table 1.

The surfactants gave rise to an interesting effect on the property of the complex substance (TB-ASA)-BSA. In a weak acid medium, the hydrogen ion can strongly have an effect on the existing state for the ion type surfactants. These characteristic groups can strongly interact with the compound formed between BSA and TB-ASA. When the concentration of an ion type surfactant such as CTMAB or SDS is lower, the micelles formed can increase the solubility and stability of the system. However, in a neutral surfactant medium such as Tween-80, OP, the micelles formed by the surfactant can obviously increase the solubility and stability of the compound. The reason is that in this kind of medium the micelle concentration is larger compared with the one of cation or anion surfactants. Thus, a neutral surfactant can strongly increase the solubility of the compound formed between BSA and TB-ASA. This kind of surfactant can obviously have an effect on the compound formed between BSA and TB-ASA. Compared with Tween-80, emulgent OP has much more hydrophilic group that resulted in larger solubility of the compound in the presence of OP. The sensitivity and stability of the determination of BSA in the presence of OP is higher than the one in the presence of Tween-80 (Table 1).

Table 1. Comparison of absorbance and stability of each system.

System	λ_{max} (nm)	ΔA_{max}^{*}	Stability
Tween-80 + B-R + (TB-ASA) + C_2H_5OH + BSA	598	0.115	After the addition of Hb, the system immediately reaches stability and is stable for 40 min
SDS + B-R + (TB-ASA) + C_2H_5OH + BSA	360	0.383	After the addition of Hb, the system immediately reaches stability and is stable for 140 min
OP + B-R + (TB-ASA) + C_2H_5OH + BSA	354	0.477	After the addition of Hb, the system immediately reaches stability and is stable for 560 min

*$\Delta A_{max} = A_1 - A_2$.

Optimization of experimental conditions

The acidity experiment studies showed that the maximum absorbance was found over the range pH 2.6-3.2. Outside this range, the sensitivity of the determination of BSA is lower. The H^+ does not participate in the reaction between BSA and TB-ASA. However, the acidity has the effect of the existing state of BSA and TB-ASA. Thereby it affects the recombination between the reactants BSA and TB-ASA. In the experiments, pH 2.9 was recommended as the working pH value. The appropriate amount of B-R buffer solution was 1.5-2.5 mL and over this range the maximum and constant absorbance was gained; 2.0 mL of the buffer was suitable in a final volume of 10 mL.

The effect of the amount of TB-ASA on absorbance was observed by determining different concentration of TB-ASA under the optimum conditions. The results showed that with increase in the amount of TB-ASA the absorbance gradually raised. When the amount of TB-ASA solution reached 1.0 mL and the concentration of TB-ASA was 5.0×10^{-5} M, the absorbance was at maximum. 1.0 mL of 5.0×10^{-4} M TB-ASA solution was selected in the experiments.

A different volume of ethanol was added, and the results showed that the absorbance increased obviously as the addition amount of ethanol increased. This indicated that ethanol could effectively enhance the sensitivity of the system. The reason why ethanol can increase the sensitivity of the system is considered that after the addition of ethanol ethanol-water medium is advantageous to the combination between the BSA and TB-ASA and the solubility of the compound can be increased. In the experiment, 2 mL of ethanol was selected as a suitable amount in a final volume of 10 mL.

The effect of ionic strength on the sensitivity of the system was observed by adding sodium chloride solution. The results showed that the absorbance gradually increased with the increase in ionic strength over the range of 0.1-0.5 $g \cdot L^{-1}$ of sodium chloride. Then it gradually tended to smooth and steady. When the concentration of sodium chloride was over the range of 0.5-1.0 $g \cdot L^{-1}$, the absorbance had little change over this range and had not great effect on the system.

The addition order of reagent has a strong effect on the stability and sensitivity of the system. The experimental results showed that the addition order OP-(B-R)-(TB-ASA)-C_2H_5OH-BSA is best. The emulgent OP and the buffer should be added before other reagents. In this order the sensitivity is high and the stability is good. The results of other addition order were not ideal.

Stability of system

Under the optimum experimental conditions the absorbance attained a maximum and the system was stable at room temperature 25 ± 5 °C. The variation of absorbance difference was less than 5 % within 9 h 20 min.

Effect of coexisting substance

The influence of the substances that may cause interference on the determination of protein was examined under the optimum experimental conditions. The most commonly encountered ions were added individually to a solution containing 40 mg· L^{-1} of BSA in a 10 mL of solution, the tolerance limits (weight multiple, w/w) of the common ions tested (causing < ± 5 % relative error) are summarized as follows: Fe^{2+} (0.05), Fe^{3+} (0.03), Al^{3+} (0.03), Mg^{2+} (0.3), Cu^{2+} (0.005), Ca^{2+} (0.02), Mn^{2+} (0.01), Pb^{2+} (0.02), Zn^{2+} (0.01), citric acid (0.6), ascorbic acid (0.8), urea (0.6), tartaric acid (0.2), glucose (0.1), L-lysine (0.01), L-phenylalanine (0.1), L-serine (0.07), L-tryosine (0.15), L-proline (0.15), L-tryptophane (0.3).

Calibration curve

A calibration curve for BSA was constructed under the optimum conditions. A solution containing 0, 25, 50, 100, 150, 300, 450, 600, 750 μg of BSA was transferred into a series of 10 mL calibrated flasks. Then, the operation was made according to the analytical procedure. Beer's law was obeyed in the range of 5.0-75.0 mg·L^{-1}. The linear regression equation of calibration curve is: ΔA = 0.0089C + 0.0180(C: mg·L^{-1}), with a regression (or correlation) coefficient γ = 0.9990. The apparent molar absorptivity of the method is $\varepsilon_{354\ nm}$ = 6.13 x 10^5 M^{-1}·cm^{-1}. According to the literature [8], the limit of detection is defined as $C_L = 3S_B/m$, where C_L, S_B and m are the limit of detection, standard deviation of the blank signal and slope of the calibration graph, respectively. The detection limit of the protein determination was found to be 35 ng·mL^{-1}. The relative standard deviation for eleven replicate determinations of 35 mg·L^{-1} BSA was 0.87 %.

Analysis of sample

10 mL of human serum samples were obtained from the hospital of our university. The samples were stored at about 4 °C in a refrigerator until analysis was made. 1.0 mL of the sample was taken out and diluted 100 fold with water as a testing solution for the analysis of protein. The testing solution was kept in a refrigerator at about 4 °C until the determination of protein content was conducted. When the analysis of protein in the human serum was made, 1.0 mL of the above working solution was taken out as the analytical sample solution. The rest of procedure is the same as that of standard procedure. The analytical results are given in Table 2.

The recovery test was made according to the following procedure. Into a 10 mL calibrated flask were successively placed 2.0 mL of 0.50 % (w/v) emulgent OP solution, 2.0 mL of B-R buffer solution of pH 2.9, 1.0 mL of 5.0 x 10^{-4} M TB-ASA solution, 2.0 mL of absolute ethanol, 1.0 mL of the test solution and 0.40 mL of 500 mg·L^{-1} BSA standard solution**.** Then determination of BSA was carried out according to the standard procedure. The total amount of BSA was calculated according to the linear regression equation. The total amount, which was obtained from the above-calculation, subtracts the amount in the human serum testing solution to obtain the amount of the recovered BSA. This value was divided by the added amount of BSA to obtain the recovery. The results showed that the recovery of the method was in the range 96.90-103.2 %. The relative standard deviation of five determinations of protein was in the

range 2.1-3.4 %. The results obtained by the present method are in excellent agreement with those obtained by *p*-acetylchlorophosphonazo spectrophotometric method [9].

In conclusion, the application of TB-ASA in the presence of emulgent OP as a spectroscopic probe for protein has the advantages of operation simplicity, rapidity, high sensitivity, good selectivity over existing methods. The present method is suitable for the routine analysis of protein in some biological samples for clinical analysis.

Table 2. Analytical results of samples.

Sample	BSA found ($mg \cdot L^{-1}$)	Average ($mg \cdot L^{-1}$) (*p*-cetylchlorophosphonazo –spectrophotometry [8])	BSA added ($mg \cdot L^{-1}$)	Sum of BSA found ($mg \cdot L^{-1}$)	Recovered $mg \cdot L^{-1}$ (Recovery,%)	Relative standard deviation (%)
No. 1	37.08 38.00 39.26 35.82 37.20	37.47 (37.47)	20.00	58.11	20.64 (103.2)	3.4
No. 2	45.13 44.90 45.59 42.83 44.55	44.60 (44.59)	20.00	63.98	19.38 (96.90)	2.1

REFERENCES

1. Bradford, M.M. *Anal. Biochem.* **1976**, 72, 248.
2. Wei, Y.J.; Li, K.A.; Tong, S.Y. *Talanta* **1996**, 43, 1.
3. Wei, Y.J.; Li, K.A.; Tong, S.Y. *Chem. J. Chin. Univ.* **1996**, 17, 1687.
4. Li, H.C.; Gao, J.H. *Chin. J. Spectr. Lab.* **2004**, 21, 112.
5. Dourmas, B.T.; Watson, W.; Biggs, H.G. *Clin.Chim. Acta* **1971**, 31, 87.
6. Schosinsky, K.H.; Vargas, M.; Eoqulevl, A.L.; Chavarria, M.A. *Clin. Chem.* **1987**, 33, 223.
7. Wu, F.X.; Yu, X.M. *Metallu. Anal. (in Chinese)* **1984**, 4, 6.
8. Motolla, H.A. *Kinetic Aspects of Analytical Chemistry*, Wiley: New York; **1998**, p 99.
9. Zhai, Q. Z.; Zhang, J. *Chin. J. Anal. Lab.* **2005**, 25, 55.