Asian Journal of Chemistry Vol. 20, No. 7 (2008), 5060-5066

Spectrophotometric Determination of Hemoglobin with Lanthanum(III)-(DBC-Arsenazo)

QING-ZHOU ZHAI* and XIAO-XIA ZHANG
Research Center for Nanotechnology, South Campus, Changchun University of Science and Technology, 7186 Weixing Road, Changchun-130022, P.R. China
E-mail: zhaiqingzhou@sohu.com; zhaiqingzhou@hotmail.com

A spectrophotometric determination of hemoglobin was proposed with lanthanum(III)-(DBC-arsenazo) complex as a spectrum probe in the presence of emulgent OP. In a Britton-Robinson buffer at pH 2.9, hemoglobin, La(III) and DBC-arsenazo form a colour compound. At its maximum absorption wavelength of 364 nm, the apparent molar absorptivity for the determination of hemoglobin is $\varepsilon_{364\ nm} = 3.59 \times 10^5$ L mol^{-1} cm^{-1}. The linear relationship between the concentration of hemoglobin and its absorbance exists over the concentration range of 10-70 mg L^{-1} of hemoglobin and Beer's law is obeyed. The proposed method was directly used in the determination of total proteins in human serum samples with satisfactory results.

Key Words: Lanthanum(III), DBC-Arsenazo, Hemoglobin, Spectrophotometry.

INTRODUCTION

Protein is a necessary nourishment component for human body and closely related to growth, heredity, metabolism life activities, *etc*. In biochemistry and clinical analysis, protein is a very important test item. Thus, the determination of protein is very important for the evaluation of the health of human body. Because most of proteins are colourless and have not any absorption over the visible light region, spectrum probes are often used for the determinations of proteins. Lowry *et al.*[1] and Bradford[2] methods are classical ones for the determination of protein. However, these methods have the disadvantages of low sensitivity, poor anti-disturbance ability and great difference of measurement between different proteins. Although some spectrophotometric methods for the determination of protein such as bromophenol blue[3], arsenazo K[4] spectrophotometry have been proposed, the selectivity of these methods is still poor. Some metal ion-dye probes, dibromohydroxyphenylfluorone-molybdenum(VI)[5], bromopyrogallol red-zinc(II)[6] have already been used for the determination of proteins. However, the sensitivity and selectivity are not ideal. Development of a new method for the analysis of protein is still necessary.

DBC-arsenazo (DBC-ASA, dibromo-*p*-chloro-arsenazo)-2,6-dibromo-4-chlorophenylazo)-4,5-dihydroxy-2,7-naphthalene disulfonic acid[7] has been used for the determination of rare earths by spectrophotometry. Here, the rare earth complex (DBC-ASA)-La(III) is applied as a new spectroscopic probe for hemoglobin and a new method for the determination of protein was established. The present method was successfully used in the determination of the total proteins in human serum samples with satisfactory results. Compared with the classical Lowry method[1], the present method is free of heating procedure and the operation is simpler and more convenient. Compared with Bradford method[2], the present procedure has the advantages of high sensitivity, wide linear range and operation simplicity, *etc*.

EXPERIMENTAL

Hemoglobin (Hb, Shanghai Lizhu Dongfeng Biotechnology Co., Ltd., Shanghai, China) working solution (400 mg L^{-1}) was prepared by dissolving 0.0400 g of Hb in 100 mL of deionized water and stored at 0-4 ºC. The aqueous DBC-arsenazo (DBC-ASA, $C_{22}H_{14}N_4O_{11}S_2Br_2ClAs$, Shanghai Changke Research Institute for Reagent) solution (2×10^{-4} mol L^{-1}) was prepared by dissolving 0.0169 g of DBC-ASA in 100 mL of deionized water. A La(III) working solution of 5.0×10^{-3} mol L^{-1} was prepared by dissolving 0.1629 g of La_2O_3 (spectroscopically pure) in 2 mL of hydrochloric acid (1+1, v/v) and diluted to 100 mL with deionized water. A 0.5 % (w/v) OP emulgent (The Fifth Chemical Plant of Shengyang, China) solution was prepared by dissolving 0.500 g of OP in 100 mL of deionized water. A pH 2.9 Britton-Robinson (B-R) buffer solution was obtained by mixing 17.5 mL of 0.2 mol L^{-1} NaOH with 100 mL of mixed-acid solution containing phosphoric acid, acetic acid and boric acid each with a concentration of 0.04 mol L^{-1}. Absolute ethanol (The Fifth Chemical Plant of Shenyang, China) was used. All reagents were of analytical reagent grade and doubly deionized water was used throughout.

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

Standard procedure: The following components were mixed in a 10 mL volumetric flask: 1.0 mL of 0.50 % (w/v) OP solution, 1.5 mL of B-R buffer solution of pH 2.9, 0.20 mL of 2×10^{-4} mol L^{-1} DBC-ASA solution, 2 mL of absolute ethanol, 0.50 mL of 5×10^{-3} mol L^{-1} La(III) solution and 1 mL of 400 mg L^{-1} Hb solution. The mixture was diluted to the mark with water, mixed thoroughly and kept at room temperature (15- 25 ºC) for 10 min. The absorbance (A) of the solution was measured at 364 nm against water. The absorbance (A_0) of the blank sample without hemoglobin was

obtained under the same conditions. Then, the absorbance difference ($\Delta A = A\text{-}A_0$) 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

The absorption spectra are shown in Fig. 1. It can be seen from the curve A that the maximum absorption of DBC-ASA against water locates at 512 nm. The maximum absorptions of other system (Curve b-e) against water are at 364 nm. The violet movement of the absorption wavelength indicates the formation of a new complex. It can be seen from curve B and D that the addition of Hb made the increase in absorbance at 364 nm. This indicates that a new compound formed. The addition of ethanol can further increase the absorbance of the coloured compound. Thus, Hb-(DBC-ASA)-La(III)-OP-C_2H_5OH system is selected for the determination of Hb. The maximum absorption wavelengths of Hb-(DBC-ASA)-La(III)-OP-C_2H_5OH and the corresponding reagent blank (DBC-ASA)-La(III)-OP-C_2H_5OH against water are observed at 364 nm. The maximum difference of absorbance is observed at 364 nm. In the following experiments, the measurement wavelength is selected to be 364 nm.

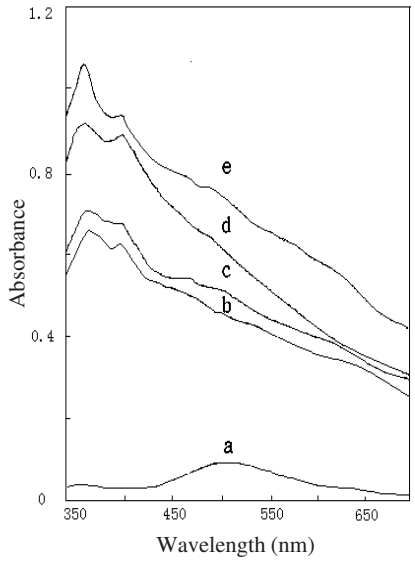

Fig. 1. Absorption spectra: (a) DBC-ASA (against water); (b) (DBC-ASA)-La(III)-OP (against water); (c) (DBC-ASA)-La(III)-OP-C_2H_5OH (against water); (d) Hb-(DBC-ASA)-La(III)-OP (against water); (e) Hb-(DBC-ASA)-La(III)-OP-C_2H_5OH (against water); Experimental conditions: pH 2.9, [Hb] = 40 mg L^{-1}, [DBC-ASA] = 4.0×10^{-6} mol L^{-1}, [La(III)] = 2.5×10^{-4} mol L^{-1}, [OP] = 0.050 % (w/v), [C_2H_5OH] = 20 % (v/v)

Effect of surfactant: The effect of different kind of surfactants was observed. In the absence of a surfactant a coloured complex was immediately precipitated. After the surfactant OP emulgent, Tween-80, cetyltrimethyl-ammonium bromide (CTMAB) or dodecyl sulphate (SDS) was added, the stability and sensitivity of corresponding coloured system are obviously improved and raised. The absorbance and stability of each system are listed in Table-1. Although the addition of Tween-80 can make system stable, the stable time is only 30-40 min. The addition of CTMAB makes the sensitivity and stability of the system increase to a little extent. The addition of SDS could makes the sensitivity of the system enhance, but the stability of system is not good and the stable time is only for 40 min. By a comprehensive comparison of sensitivity, stability and operation convenience, the system of the addition of OP was selected. The addition of OP can make the coloured system immediately stable and the stable time of the system increases to be 2.5 h. The maximum absorbance was observed upon the addition of 0.80-1.20 mL of 0.50 % (w/v) OP emulgent solution in a final volume of 10 mL. In the experiments, 1 mL was selected to be an appropriate amount.

TABLE-1
COMPARISON OF ABSORBANCE AND STABILITY OF EACH SYSTEM

System	λ_{max}	ΔA_{max}	Experimental phenomena
(B-R)-(DBC-ASA)-La(III)-Hb	/	/	After the addition of Hb, the precipitation immediately appears
OP-(B-R)-(DBC-ASA)-La(III)-C_2H_5OH-Hb	364	0.490	After the addition of Hb, the system immediately reaches stability and is stable for 2.5 h
SDS-(B-R)-(DBC-ASA)-La(III)-C_2H_5OH-Hb	396	0.443	After the addition of Hb, the system immediately reaches stability and is stable for 40 min
CTMAB-(B-R)-(DBC-ASA)-La(III)-C_2H_5OH-Hb	400	0.303	After the addition of Hb, the system immediately reaches stability and is stable for 20 min
Tween-80-(B-R)-(DBC-ASA)-La(III)-C_2H_5OH-Hb	396	0.209	After the addition of Hb, the system immediately reaches stability and is stable for 0.5 h

Effect of acidity: The effect of acidity was observed over the pH range of 2.1-4.1. The results showed that ΔA increases with increasing pH value over the pH range of 2.1-2.6. The maximum ΔA was attained over the pH range of 2.6-3.3. Thus, pH 2.9 was selected in the experiments. The maximum and constant ΔA remains over 0.50-2.0 mL of B-R buffer solution. In the experiments, 1.5 mL of the buffer was suitable in a final volume of 10 mL.

Effect of the chormogenic reagent: For the determination of 40 mg L^{-1} Hb, ΔA remains maximum when the amount of DBC-ASA is over the range of 0.15-0.50 mL. A 0.20 mL of 2×10^{-4} mol L^{-1} DBC-ASA was selected in the experiments.

Effect of the amount of lanthanum(III): The effect of the amount of lanthanum(III) on absorbance was studied for 40 mg L^{-1} Hb. The results show (Fig. 2) that with the increase in the amount of La(III), the absorbance enhances. When the amount of La(III) was 0.50 mL, the absorbance was maxmium. Hence, 0.50 mL was recommended in the experiments.

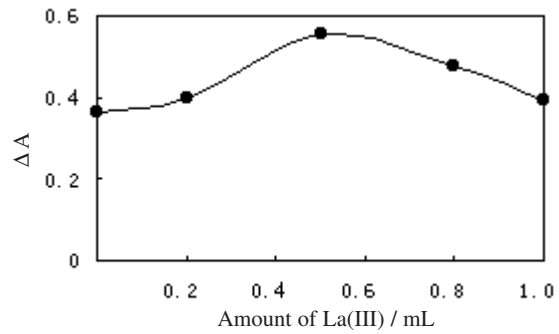

Fig. 2. Effect of the amount of lanthanum(III): pH 2.9, [Hb] = 40 mg L^{-1}, [DBC-ASA] = 4.0×10^{-6} mol L^{-1}, [OP] = 0.050 % (w/v), [C_2H_5OH] = 20 % (v/v)

In weak acidic medium, the La(III)-(DBC-ASA) was used as a spectrum probe to react with the protein. The -OH group of DBC-ASA and La(III) form a blue-violent coordination complex. Because Cl^- group in DBC-ASA combines with -NH_3^+ of the Hb to form a compound. The intervention of La(III) does not compete the same kind of anion with the Hb; on the contrary; it makes the coloured system more stable and selective.

Effect of ethanol: A different volume of ethanol was added and the results indicated that the absorbance increased obviously as the addition amount of ethanol increased. In the experiment, 2 mL of ethanol was selected to be an appropriate amount. At this time the sensitivity can be increased by 46.5 %. The addition of ethanol can accelerate the combination between the protein and DBC-ASA. The effect result of acetone on the coloured system showed that the sensitivity can be increased by 20 % after the addition of 2 mL of acetone. Considering the toxicity and price, ethanol was selected in the experiment.

Effect of the addition order of reagents: The results of the addition order of reagent showed that according to the following addition order OP-(B-R)-(DBC-ASA)-C_2H_5OH-La(III)-Hb the best results were obtained. The sensitivity of the determination of Hb is highest and the system is most stable. The emulgent OP and buffer should be added before other reagents.

Effect of ionic strength: Sodium chloride was used to observe the effect of ionic strength on the sensitivity of system. The results indicated that the absorbance of the coloured system gradually reduced over the concentration range of 0-1.0 g L^{-1} with increasing the concentration of NaCl. When the concentration of sodium chloride increased to be 1.0 g L^{-1}, the sensitivity of the determination of hemoglobin decreased by 34 %. The combination between the Hb and DBC-ASA is mainly dependent on electrostatic conjugation. In an acid solution, the residue of the amino acid of protein carries positive charge and can combine with the anion of the DBC-ASA by electrostatic bound. As the concentration of sodium chloride increased, ΔA gradually decreased. Due to the increase in the concentration of sodium chloride, the anion of the DBC-ASA and chloride ion compete the reaction with the Hb, resulting in the decrease in the combination role between the Hb and the dye.

Composition ratio of compound: The composition ratio of the compound was determined to be La(III):(DBC-ASA):Hb::1:3:38 by both mole ratio and equimolar continuous variation methods.

Calibration curve: The different amount of standard solution of Hb was placed into a series of 10 mL volumetric flask, respectively and then the remainder procedure and the absorbance measurement were made according to general procedure. The experimental results showed that Beer's law was obeyed over the range of 10-70 mg L^{-1} of Hb. The linear regression equation of calibration curve is: $\Delta A = 0.0085C + 0.0514$ (C mg L^{-1}), with a regression coefficient $\gamma = 0.9960$. The apparent molar absorptivity of the method is $\varepsilon_{364\ nm} = 3.59 \times 10^5$ L mol^{-1} cm^{-1}. The relative standard deviation for eleven replicate determinations of 40 mg L^{-1} of Hb was 0.98 %. The detection limit (3σ) for Hb was 85 ng mL^{-1} (here s represents the standard deviation of eleven blank measurements).

Influences of foreign substances: The influence of various ions and substances was determined. For 40 mg L^{-1} of Hb, the following species at the given ratios(multiple in mass, m/m), respectively, caused the change of relative error less than ± 5 %: Co^{2+} (2); La^{3+}, Eu^{3+} (0.5); Ba^{2+}, Bi^{3+} (0.2); Al^{3+} (0.1); Ca^{2+} (0.07); Mg^{2+} (0.05); Zn^{2+}, Ti^{4+} (0.02); Fe^{3+}, Cu^{2+}, Cd^{2+}, Pb^{2+}, Fe^{2+} (0.01); Mn^{2+} (0.002); Cl^- (20); F^-, NO_3^- (10); Br^- (1); NO_2^- (0.5); MnO_4^-, WO_4^-, VO_3^- (0.1); SiO_3^{2-} (0.01); I^- (0.001); oxalic acid (10), ascorbic acid (1); tartaric acid, EDTA (ethylene diamine tetraacetic acid, 0.1); L-lysine, L-tryosine, L-phenylalanine, tartaric acid (0.05); urea (0.02).

Analysis of sample: The human serum samples were taken from the hospital of Changchun University of Science and Technology. The testing solutions were obtained by the dilution of 100 fold of the above solutions with water for the analysis of protein. 1 mL of the above testing solution was taken out as the analytical sample solution and the remainder is the

same as the standard procedure for Hb assay. The recovery tests were followed by the standard addition method. The analytical results are shown in Table-2. It can be seen from the Table that the analytical results obtained are in excellent agreement with those obtained by the *p*-acetylchlorophosphonazo-barium(II) method[8]. The recoveries of the method are between 99-102 %. The relative standard deviation of five replicate determinations is less than 3 %. The analytical results are quite satisfactory.

TABLE-2
ANALYTICAL RESULTS OF SAMPLES

Sample	Hb found (mg L^{-1})	Average (mg L^{-1})	Hb added (mg L^{-1})	Hb recovered (mg L^{-1})	Recovery (%)	RSD (%)	Contrast method [Ref. 8]
1	32.19, 32.66, 33.01, 32.78, 32.42	32.61	20.0	20.4	102.0	0.97	32.61
2	23.25, 22.89, 23.72, 23.60, 23.25	23.34	20.0	19.8	99.0	2.05	23.33

In conclusion, the lanthanum(III)-(DBC-arsenazo) complex was used as a spectroscopic probe for the determination of hemoglobin. The developed method has the advantages of operation simplicity and high sensitivity. The linear range of the method is wide and the detection limit (85 ng mL^{-1}) is low. The present procedure has been satisfactorily applied to the determination of proteins in human serum samples with satisfactory results.

REFERENCES

1. O.H. Lowry, N.J. Rosebrough and A.L. Farr, *J. Biol. Chem.*, **193**, 265 (1951).
2. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
3. Y.J. Wei, K.A. Li and S.Y. Tong, *Talanta*, **43**, 1 (1996).
4. Q.L. Hu, F.L. Zhao and K.A. Li, *J. Instru. Anal.*, **19**, 45 (2000) (In Chinese).
5. Z.X. Guo, Y.M. Hao and X. Cong, *Anal. Chim. Acta*, **403**, 225 (2000).
6. K. Zhu, K.A. Li and S.Y. Tong, *Anal. Lett.*, **29**, 575 (1996).
7. H. Li, R.X. Cai and X.M. Yu, *Chin. J. Anal. Chem.*, **13**, 651 (1985).
8. Q.Z. Zhai and J. Zhang, *Chin. J. Anal. Lab.*, **24**, 55 (2005).

(*Received*: 4 April 2007; *Accepted*: 5 April 2008) AJC-6502