Polarographic immunoassay coupled with catalysis of non-radioactive multiple iodine label*

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Abstract A new polarographic immunoassay was developed. In this assay, human serum albumin (HSA) as the model antigen was covalently labeled with organic compound erythrosin B (EB) containing four non-radioactive iodides through 1-step chemical reaction. The labeling procedure is simple and the conditions needed are moderate. The molar labeling ratio of EB:HSA was 12:1. The content of iodine in the conjugate obtained by the proposed procedure is much higher than that by the other existing methods. A heterogeneous competitive immunoassay was established by coupling the catalysis of the conjugate to substrate As(Ⅲ)-Ce(Ⅳ) reaction with the linear-sweep polarographic detection of As(Ⅲ) amount. HSA can be determined in the HSA concentration range from 1 to 200 μg/mL, with the detection limit of 0.66 μg/mL.

Keywords: HSA, erythrosin B, immunoassay, catalysis, linear-sweep polarography.

Immunoassay is one of the powerful tools in medical research and clinical examination. Radioimmunoassay (RIA) and enzyme-linked immunoassay (EIA) have been widely used. These assays have some inherent limitations. Therefore, developing an immunoassay with either non-radioactive or non-enzyme labels is of much significance.

O'Kennedy *et al*.[1] described an indirect immunoassay for the determination of IgG in the mouse serum. In the immunoassay, IBHR reagent (3-(3-iodine-4-hydroxyphenyl) proprionic acid N-hydroxy succinimide ester) with a non-radioactive iodine was used to label antibody. The obtained iodine-containing label was determined by spectrophotometric measurement of the conversion ratio of the Ce(Ⅳ) amount by means of its catalysis to As(Ⅲ)-Ce(Ⅳ)[1], Sb(Ⅲ)-Ce(Ⅳ)[2] substrate reactions.

Reported in this paper is a new system of polarographic immunoassay. In the new system, organic reagent erythrosin B (EB) containing four non-radioactive iodides was used not only as the binding reagent but also as the marker of the label. The labeling procedure with the aid of an activated ester was very simple with only moderate conditions needed. It can be carried out in a general chemical laboratory without using special equipment. The conjugate obtained by the proposed approach is stable and easy for storage, and has the highest iodine content in comparison with existing methods. Moreover, HSA, as the model antigen, was labeled with EB. By coupling the catalysis of the EB-labeled HSA conjugate to As(Ⅲ)-Ce(Ⅳ) reaction with the linear-sweep polarographic detection of the residual As(Ⅲ) amount, a heterogeneous competitive immunoassay

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for the determination of HSA was established. The calibration plot for HSA was obtained in the concentration range from 1 to 200 μg/mL. The detection limit was 0.66 μg/mL. The proposed immunoassay was used to determine HSA level in human serum samples. The proposed system offers potential prospects for the labeling and assay of active proteins.

1 Experimental

1.1 Apparatus

A Model JP-2 linear-sweep polarograph (Chengdu Instrumental Factory, Chengdu, China) was equipped with a three-electrode system which involves a dropping mercury working electrode, a Ag/AgCl reference electrode and a Pt wire auxiliary electrode. The three-electrode system was minimized to be inserted directly into the well of microtitre plate for polarographic measurement, as described in ref. [3]. A Model LC-6A high performance liquid chromatograph (Shimadzu Co., Japan) was equipped with a hydrophobic interaction column (4 mm i.d. ×150 mm). A Model PE17 UV/Vis spectrophotometer (PE Co., USA) was used.

1.2 Materials

Human serum albumin (HSA, purity > 99%) and sheep anti-human HSA serum (titre 1:40) were purchased from the Shanghai Biological Preparation Institute. Bovine serum albumin (BSA) was purchased from the Lanzhou Biological Preparation Institute. The erythrosin B was of CP grade (Shanghai No. 3 Chemical Reagent Factory). Isoamyl chlorocarbonate (AR grade) was purchased from Fluka Co. (USA). The 3-*n*-butylamine was of AR grade (BASF). The 2, 4-dioxane of AR grade was obtained from the Xi'an Chemical Reagent Factory. All the other chemicals were of AR grade. Double distilled water was used. Spectra/por dialysis visking tubing (MWCO of 12000) was purchased from the Fisher Scientific (USA). Human serum sample was provided by The People's Hospital of Shaanxi Province.

1.3 HSA labeling

Weigh 5.2 mg of solid powder EB (molar ratio of EB:HSA was about 15:1). Add 360 μL of dioxane, 12 μL of 3-*n*-butylamine and 10 μL of isoamyl chlorocarbonate into the powder EB at ice-water bath, and let the reaction proceed for 30 min. The obtained solution was solution A. Additionally, weigh 27.2 mg of HSA. Add 750 μL of water and 500 μL of dioxane to dissolve HSA and adjust the medium pH to pH 7—9, then cool the mixture at ice-water bath. The obtained solution was solution B. Add solution A drop by drop to solution B with stirring under pH 7—9, and let the reaction proceed for 4 h at 4℃. Transfer the obtained mixture into the dialysis tubing, and dialyze against flow water for 3 h and against 0.01 mol/L phosphate buffered saline (PBS) for 48 h. Dissolve the EB-labeled HSA (EB-HSA) in 5 mL of 0.01 mol/L PBS solution to be used as the standard stock solution. The standard stock solution and the other working solution of EB-HSA were stored at 4℃.

1.4 Identification of the EB-labeled HSA

Record the UV spectra of EB, HSA and EB-HSA solutions against water at 210—386 nm by UV/Vis spectrophotometry, respectively. Measure the absorbances of these compounds at 277.8

nm and calculate the molar labeling ratio of EB:HSA in EB-HSA according to their molar absorption coefficients.

Record high performance hydrophobic interaction liquid chromatograms of EB, HSA and EB-HSA, respectively. The chromatographic conditions were as follows: Mobile phase A was 3 mol/L $(NH_4)_2SO_4$-0.05 mol/L KH_2PO_4 (pH = 7.0) solution. Mobile phase B was 0.05 mol/L PBS (pH = 7.0) solution. Gradient elution from 100% phase A to 100% phase B was completed within 35 min. The flow rate was 1 mL/min. The detector was a 280-nm UV detector.

1.5 Procedure of competitive heterogeneous immunoassay

Titre 1:1 000 of sheep anti-human HSA serum was obtained by diluting the antiserum with 0.05 mol/L $NaHCO_3$-Na_2CO_3 (pH = 9.6) buffer solution. 200 μL of the diluted antiserum solution was added into the well of a microtitre plate, and was incubated for 1 h at 37℃ and then overnight at 4℃. The well was rinsed 3—5 times with PBS containing 0.05% Tween 20 (PBST). 200 μL of the mixture of the given amount of EB-HSA and standard HSA or serum sample for analysis were added into the well, which was incubated for 2 h at 37℃ and rinsed 3—5 times with PBS. 100 μL of 2.33×10^{-4} mol/L As(Ⅲ)-1.25×10^{-3} mol/L Ce(Ⅳ)-H_2SO_4 (pH = 1.4 ± 0.2) substrate solution was added into the well and incubated for 1 h at 37℃. The microtitre plate was quickly cooled to room temperature. Then the electrode-minimized system of model JP-2 linear-sweep polarograph was directly inserted into the well. The initial potential was −0.40 V. The potential was cathodically scanned. The first derivative peak current of the residual As(Ⅲ) was recorded. The HSA contents in human serum samples were determined by generating the calibration curve of HSA concentration vs. the peak current of As(Ⅲ).

2 Results and discussion

2.1 Labeling principle

There are four iodines and one carboxylic group in an EB molecule. The carboxylic group can react with isoamyl chlorocarbonate in the presence of 3-*n*-butylamine to produce an activated ester, which can react with the free amino acid residual in protein. Consequently, the EB molecule was directly linked to HSA through amide bond. The labeling procedure is shown in fig. 1. In comparison with the IBHR method, all chemicals used in the proposed method are stable and easy to be obtained. The labeling procedure was very simple and the condition was moderate.

2.2 Characterization of EB-HSA

The high performance liquid hydrophobic interaction chromatograms of EB, HSA and EB-HSA by gradient elution method are shown in fig. 2. From fig. 2, the retention times of EB, HSA and EB-HSA in the column are 0, 14.4 and 23.11 min, respectively. The EB-HSA gave only a single peak, indicating that the labeling of HSA with EB by using the proposed procedure was complete and equal, and the purification of EB-HSA was thorough going.

UV spectrophotometric analyses of EB, HSA and EB-HSA showed that HSA has a specific absorption maximum at 277.6 nm, and there are absorptions of EB and EB-HSA at the same wave-length as well. According to the addition principle of absorbance, the mole number of EB

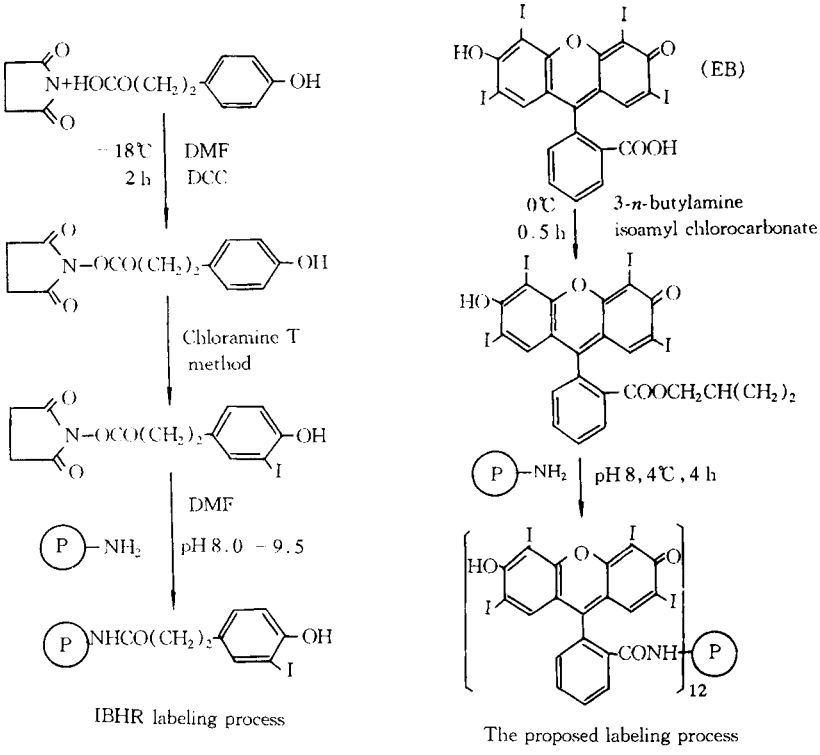

Fig. 1. Comparison of IBHR method and the proposed method.

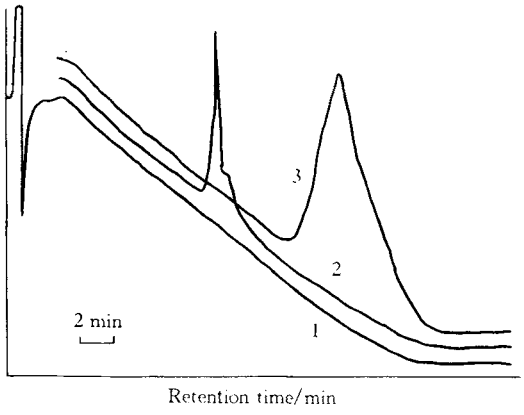

Fig. 2. HPHIC chromatograms of EB (curve 1), HSA (curve 2) and EB-HSA (curve 3) with injection of 200 μg of sample.

labeled to an HSA molecule was calculated[4]. The results are shown in table 1. In general, it is more perfect to covalently bind about 10 labeling markers per molecule of protein without significantly altering the immune activity of the labeled protein. From table 1, 12 EB molecules were conjugated to an HSA molecule. 12, the number of labeling markers, was approximate to 13—14 of the markers obtained by IBHR method[5,6]. Because an EB molecule contains four iodides, the iodine-labeled number per HSA increased nearly four times, which would improve the sensitivity of the immunoassay.

Table 1 Number of EB molecules labeled to HSA

Name	Molar mass	Concentration/μg·mL^{-1}	Absorbance	EB number
EB	835.9	15.3	0.284	–
HSA	65 000	500	0.209	–
EB-HSA	–	500	1.433	12

2.3 Optimization of the immunoassay conditions

2.3.1 Titration of anti-HSA serum with EB-HSA. According to the requirement of competitive immunoassay, titration of various titres of the precoated antiserum by using various dilution ratios of EB-HSA was carried out. Titration curves are illustrated in fig. 3. From fig. 3, it can be seen that when the dilution ratios of EB-HSA were 1 : 50 and 1:100, respectively, the peak current of residual As(Ⅲ) was kept higher and changed little, indicating that the concentration of EB-HSA was less than the effectual binding sites of the precoated antiserum. When the dilution ratios of EB-HSA were 1:10 and 1:20, respectively, the peak current of residual As(Ⅲ) increased gradually with increasing the titre of antiserum. When the titre of the precoated antiserum was higher than 1:1 000, the peak current of residual As(Ⅲ) tended gradually to the maximum value. The saturation of the precoated antibody binding sites was approached. So, the titre 1:1 000 of the antiserum and the dilution ratio 1:20 of EB-HSA were used. Additionally, for the competitive equilibrium of immune reaction in the assay, the incubate time was 2 h, and the incubate temperature was 37℃.

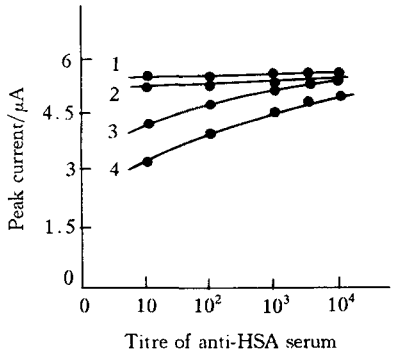

Fig. 3. Titration curves of the precoated anti-HSA serum with EB-HSA dilution ratio of EB-HSA. 1, 1:100; 2, 1:50; 3, 1:20; 4, 1:10.

2.3.2 Composition of substrate and polarographic detection. The determination of As(Ⅲ) amount by using polarography is superior to that of Ce(Ⅳ) amount by spectrophotometry in sensitivity. In pH 1.4 ± 0.2 H_2SO_4 medium, there is no As(Ⅴ) reduction wave in cathodic potential range. As(Ⅴ) does not interfere the determination of As(Ⅲ). As(Ⅲ) can yield only one polarographic reduction wave. The peak potential is -0.78 V. The first derivative peak current of As(Ⅲ) is linearly proportional to As(Ⅲ) concentration in a larger range. The relationship between the peak current and As(Ⅲ) concentration is shown in table 2.

Table 2 Relationship of As(Ⅲ) concentration with its peak current

Concentration range of As(Ⅲ) $\times 10^{-4}$/mol·L^{-1}	Linear regression equation		
	intercept/μA	slope $\times 10^4$/μA·mol^{-1}·L	corr. coeff. r
0.116—1.16	−0.3	8.2	0.997
1.16— 2.67	−0.3	7.3	0.998

When As(Ⅲ) concentration was 2.33×10^{-4} mol/L in the AS(Ⅲ)-Ce(Ⅳ) substrate solution, the effect of the [Ce(Ⅳ)]/[As(Ⅲ)] concentration ratio on the As(Ⅲ) peak current was examined. The results are shown in table 3. From table 3, it is seen that when the [Ce(Ⅳ)]/[As(Ⅲ)] concentration ratio was between 2.2 and 6.5; that is, Ce(Ⅳ) concentration was in the range from 0.5 to 1.5×10^{-3} mol/L, the effect was little. Therefore, the optimal substrate solution was 2.33×10^{-4} mol/L As(Ⅲ)-1.25×10^{-3} mol/L Ce(Ⅳ)-H_2SO_4 (pH = 1.4 + 0.2) solution. In this optimal substrate solution, the [Ce(Ⅳ)]/[As(Ⅲ)] concentration ratio was higher than 5, so the rate of As(Ⅲ)-Ce(Ⅳ) reaction catalyzed by EB-HSA is not related to the Ce(Ⅳ) concentration[7]. However, the change of As(Ⅲ) amount is closely related to the amount of EB-HSA.

Table 3 Effect of [Ce(Ⅳ)]/[As(Ⅲ)] ratio on the peak current of As(Ⅲ)

Ce(Ⅳ) concentration $\times 10^{-3}$/mol·L^{-1}	0.50	1.25	1.50	2.00
Ratio of [Ce(Ⅳ)]/[As(Ⅲ)]	2.2	5.4	6.5	8.7
Peak current of As(Ⅲ)/μA	9.2	8.8	8.6	7.3

2.3.3 Condition of the catalytic reaction. The catalytic ability of organic iodine-containing compound to As(Ⅲ)-Ce(Ⅳ) reaction depends on the structure, composition, concentration of the compound, and reaction temperature, time and others. As shown by experiments (fig. 4), EB of μg/mL magnitude in concentration could quickly catalyze As(Ⅲ)-Ce(Ⅳ) reaction at 16℃, and could be determined using the initial rate method in 1 min[8]. However, it took long time for EB of ng/mL magnitude in concentration to catalyze the same As(Ⅲ)-Ce(Ⅳ) reaction even at 37℃. According to the labeling number of EB in EB-HSA, ng/mL magnitude of EB corresponds to μg/L of EB-HSA. Therefore, HSA of μg/L can be theoretically determined if the reaction is incubated at 37℃ for a longer time. In this assay, the incubate temperature for catalytic reaction was 37℃ and the incubate time was 1 h.

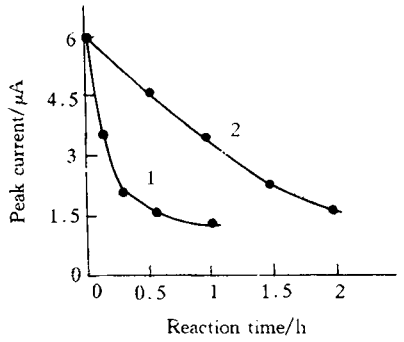

Fig. 4. Relationship of As(Ⅲ) peak current with reaction time in the presence of 440 ng/mL (curve 1) and 44 ng/mL (curve 2) EB at 37℃.

Experiments also showed that the EB-HSA of μg/mL magnitude catalyzed the reaction so slowly at room temperature as it was observed that the peak current of As(Ⅲ) was unchanged for 1 h. Therefore, after the incubation for catalytic reaction at 37℃, provided the reaction system was quickly cooled to room temperature, the catalytic reaction would be stopped. Obviously, it is suitable and convenient to assay a lot of samples simultaneously using the fix-time method for the determination of the As(Ⅲ) amount.

2.4 Calibration curve of HSA

The competitive calibration curve for HSA obtained by the proposed immunoassay precedure is shown in fig. 5. HSA was determined in the concentration range of 1—200 μg/mL. The coefficients of variation for eight analyses of 1 μg/mL and 10 μg/mL HSA were 4.1% and 2.8%, respectively. According to ref. [9], the detection limit of the proposed immunoassay is 0.66 μg/mL of HSA.

2.5 Determination of HSA in human serum samples

After 10 μL of human serum specimen was diluted to 25 mL with PBS, the HSA level was determined by the proposed immunoassay. Results obtained by the proposed immunoassay and the dye-binding spectrophotometry are listed in table 4. These results are in agreement with each other. As shown in fig. 6, the interrelation of the two results is as follows: intercept, 3.1; slope, 0.80; and the correlation coefficient r, 0.975.

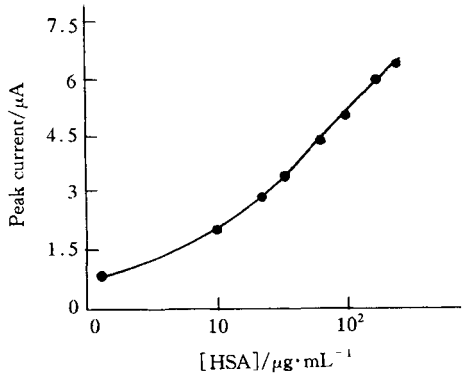

Fig. 5. Calibration curve of HSA for the competitive heterogeneous immunoassay.

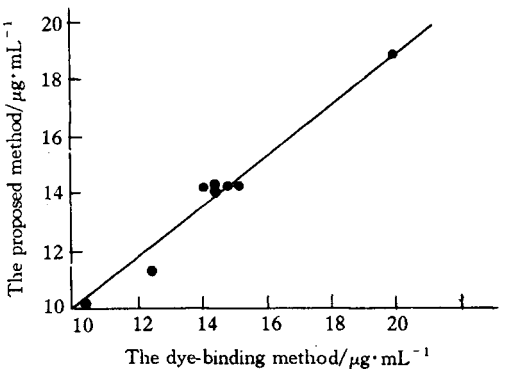

Fig. 6. Correlation of the two results obtained by the proposed immunoassay and the dye-binding spectrophotometry.

Table 4 HSA levels in human serum samples

Sample	1	2	3	4	5	6	7	8	9
Results by this assay	14.1	14.1	11.4	19.9	14.6	10.2	14.1	14.2	14.4
/μg·mL^{-1}	14.1	14.1	11.0	20.0	13.9	9.9	14.1	14.2	14.0
Results by dye-binding method/μg·mL^{-1} a)	14.4	14.0	12.4	18.8	14.4	10.4	14.8	15.2	15.2

a) Samples and results were provided by The People's Hospital of Shaanxi Province.

In conclusion, the proposed method can label and determine various proteins of interest. The polarographic immunoassay for determining ng/mL Tetanus Toxoid (TT) and μg/mL IgG has been established.

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