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Capillary electrophoretic enzyme immunoassay with electrochemical detection using a noncompetitive format

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Abstract

A capillary electrophoretic enzyme immunoassay with electrochemical detection (CE-EIA-ED) using a noncompetitive format has been developed. In this method, antigen (Ag) reacts with an excess amount of horseradish peroxidase (HRP)-labeled antibody (Ab*). The free Ab* and the bound Ag–Ab* complex produced in the solution are separated by capillary zone electrophoresis in a separation capillary. Then they catalyze enzyme substrate 3,3′,5,5′-tetramethylbenzide (TMB(Red)) and H_2O_2 in a reaction capillary following the separation capillary. The reaction product, TMB(Ox), can be determined using amperometric detection on a carbon fiber microdisk bundle electrode at the outlet of the reaction capillary. Due to the amplification of the enzyme, a significant amount of TMB(Ox) can be produced for detection. Therefore, the limit of detection (LOD) of CE-EIA-ED is very low. A tumor marker (CA15-3) was used as a model, in order to test the method. The concentration LOD of CA15-3 is 0.024 U/ml, which corresponds to a mass detection limit of 1.3×10^{-7} U.
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1. Introduction

Capillary electrophoresis (CE) is a powerful technique for the separation of macromolecules such as proteins and immunocomplexes [1]. With both superior separation power and high detection sensitivity, CE can separate free antibody or antigen from bound antibody or antigen rapidly, and is especially suitable for immunoassay [2]. The method called capillary electrophoretic immunoassay (CEIA) offers several advantages over conventional immunoassays,

such as high selectivity, low reagent consumption and short incubation time. The procedure of immunoassay can be simplified by CE separation. Many wash steps can be eliminated. In CEIA, UV detection [3,4] and laser-induced fluorescence (LIF) detection [5–17] have been used. However, the major disadvantage of the UV detection is the lack of sensitivity. The minimum detectable concentration by UV detection is around 10^{-6} mol/l. LIF detection with the minimum limit of detection of 10^{-11} mol/l [18] is a more general approach to improve sensitivity.

Amperometric detection provides excellent sensitivity for the small dimensions associated with CE, while offering a high degree of selectivity toward electroactive species. In this paper, a novel capillary

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electrophoretic enzyme immunoassay with electrochemical detection (CE-EIA-ED) using a noncompetitive format has been developed. An excess amount of horseradish peroxidase (HRP)-labeled antibody (Ab*) is added to the sample to form its bound complex (Ag–Ab*) with the antigen (Ag) present in the sample. After equilibrium is established, a small volume of incubate is injected into a separation capillary, whereupon the free Ab* and the Ag–Ab* are separated by CE, then both can catalyze the reaction of the enzyme substrate 3,3′,5,5′-tetramethyl-benzidine (TMB(Red)) and H_2O_2. The enzyme catalysis reaction proceeds in a catalysis reaction capillary following the separation capillary. The reaction products are TMB(Ox) and H_2O. TMB(Ox) can be reduced at a carbon fiber microdisk bundle electrode. Thus, the activity of HRP on the free Ab* and the Ag–Ab* can be measured by determining the reduction current of TMB(Ox) on the microdisk bundle electrode at the outlet of the catalysis reaction capillary. Since the concentration of TMB(Ox) is much higher than those of the free Ab* and the Ag–Ab* due to the enzyme amplification, LOD of CE-EIA-ED should be very low. The main advantages of CE-EIA-ED are simplicity and sensitivity besides the strong points of CEIA mentioned above.

CA15-3 (Ag) is a circulating antigen which is relatively specific for human breast tissue and is defined by two monoclonal antibodies: DF3, which is found on the surface of mammary carcinoma cells, and 115D8 which is found on milk-fat globule membrane [19,20]. CA15-3 is significantly more sensitive than carcinoembryonic antigen in the evaluation of patients with both primary and metastatic breast cancer. For healthy human, the concentration levels of CA15-3 are lower than 30 U/ml [21]. CA15-3 levels are often measured by immunoradiometric assay [22–24], enzyme immunoassay [25], microparticle enzyme immunoassay [25,26], chemiluminescence immunoassay [25] and immunofluorometric assay [27,28]. These conventional immunoassays have some shortcomings such as being time-consuming, having high reagent consumption and complicated operation. Therefore, CA 15-3 as a model in CE-EIA-ED with a noncompetitive format was investigated.

2. Experimental

2.1. Apparatus

2.1.1. CE-EIA-ED system

The CE-EIA-ED system is illustrated in Fig. 1. It consisted of six main parts, a high-voltage power supply (Model 9323HVPS, Beijing Institute of New Technology, Beijing, China) (1), a running buffer
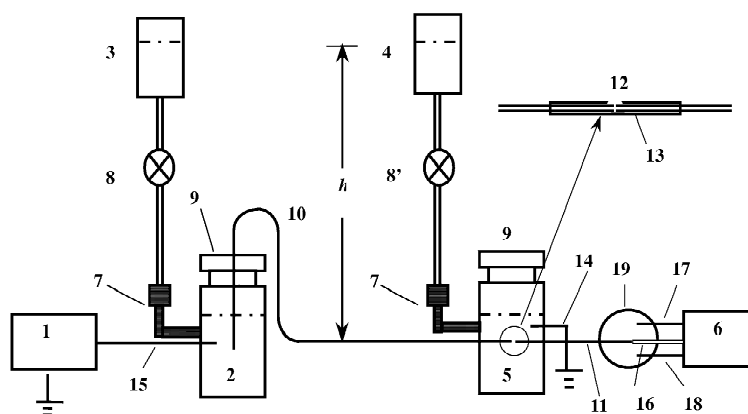

Fig. 1. Overview of the CE-EIA-ED system. 1, High-voltage power supply; 2, running buffer reservoir; 3, liquid pressure buffer reservoir; 4, liquid pressure substrate reservoir; 5, catalysis reactor; 6, electrochemical detector; 7, metal tubing; 8 and 8′, switch; 9 and 9′, rubber cover; 10, separation capillary; 11, reaction capillary; 12, hole; 13, syringe needle; 14, Pt cathode; 15, Pt anode; 16 working electrode; 17 reference electrode; 18 auxiliary electrode; 19 electrochemical cell.

reservoir (2), a liquid pressure buffer reservoir (3), a liquid pressure substrate reservoir (4), a Plexiglas catalysis reactor (5) and an electrochemical detector (6). In the system, the cylindrical running buffer reservoir (2) (12 mm diameter and 20 mm in depth) was made from Plexiglas with a rubber cover (9). There is a metal tubing (7) in the reservoir wall. The metal tubing linked up with the running buffer reservoir (2) and the liquid pressure buffer reservoir (3) through a plastic hose. On the hose, there is a switch (8) to control the flow from the liquid pressure buffer reservoir. In this system, a metal needle (13) (400 μm I.D., 680 μm O.D.) of syringe with a hole (12) in the middle passed through the catalysis reactor (5). Both the polyacrylamide-coated separation capillary (50 μm I.D., 375 μm O.D., 15 cm length) (10) and the polyacrylamide-coated re-action capillary (50 μm I.D., 375 μm O.D., 5 cm length) (11) were inserted in the needle with a gap (about 10 μm) between them. The separation capil-lary was connected to the running buffer reservoir through the rubber cover. The enzyme substrate (TMB(Red)) solution in the liquid pressure substrate reservoir (4) was introduced into the reaction capil-laries (11) through the gap by means of the liquid pressure. There is another switch (8′) to control the flow from the liquid pressure substrate reservoir (4). A platinum wire (14) served as the grounded elec-trode in contact with the substrate solution for the high potential drop across the separation capillary. Unless noted otherwise, the applied separation high-voltage was 20 kV. In the system, all the joints could be fixed with epoxy adhesive. CE and ED in CE-EIA-ED used in this work were similar to our previous description [29]. Briefly, a reversible high-voltage power supply (Model 9323HVPS, Beijing Institute of New Technology, Beijing, China) pro-vided a variable voltage of 0–30 kV across the separation capillary with its outlet at ground po-tential. ED at a constant potential was carried out with the electrochemical analyzer (Model CHI800, CH Instruments, Austin, TX, USA). The detection cell, the detector and the catalysis reactor were housed in a Faraday cage in order to minimize the interference from noise of external sources. ED was carried out with a three-electrode system. It consisted of a carbon fiber microdisk bundle electrode as the working electrode, an SCE used as the reference electrode, and a coiled Pt wire (0.3-mm diameter, 5 cm in length) placed at the bottom of the cell as the auxiliary electrode. The arrangement of the electro-chemical detection cell is illustrated in Ref. [29] in detail. Samples were injected hydrodynamically. The carbon fiber microdisk bundle electrodes used here were described previously [30]. Before use, all carbon fiber microdisk bundle electrodes were cleaned in alcohol and washed with double-distilled water for 5 min by a ultrasonicator. During electro-phoresis, the electrodes can be directly washed with alcohol and water in the detection cell.

2.1.2. Capillary treatment

The method for preparing the polyacrylamide-coated capillaries was similar to that used in Refs. [31–34]. The polyacrylamide-coated capillaries were prepared from the uncoated fused-silica capillaries 35 cm long and 50 μm I.D. The inner surface of the capillaries were first pretreated with 1 mol/l NaOH for 30 min and then flushed with water for 30 min. The silane solution adjusted to pH 3.5 by acetic acid containing 0.5% (v/v) γ-methacryloxypropyl-trimethoxysilane (Acros Organics, NJ, USA) and 0.5% (v/v) alcohol was sucked up into the capil-laries. After the reaction proceeded for 1 h at room temperature, the silane solution was removed. Then the capillary was filled with 3.5% (w/v) deaerated acrylamide solution containing 1 μl N,N,N',N'-tetra-methylethylenediamine and 2 mg potassium persul-phate per ml. After 3 h, the excess (not attached) polyacrylamide was sucked away and the capillaries were rinsed with water. After most of the water in the capillaries was removed by aspiration, they were then dried under a N_2 stream at 45 °C. Every coated capillary can be used for at least 50 runs. After the experiments, the coated capillary was washed with water and then dried by aspiration for analysis reproducibility between days.

2.2. Reagents and solutions

The CA15-3 EIA Kit (No. 200-10) was purchased from CanAg Diagnostics AB, Gothenburg, Sweden), which consisted of CA 15-3 standards (containing 0, 15, 50, 125 and 250 U/ml), and the anti-CA15-3 antibody (50 mg/l) labeled with horseradish per-oxidase (HRP). The kit was stored at 4 °C. The

breast cancer serum samples and the results detected by ELISA were provided by the hematological center at Qilu Hospital, Jinan, China. The serum samples were stored at $-20\,°C$. TMB(Red) (High Pure Grade) was obtained from Amresco Inc. (Solon, OH, USA). A stock standard solution of TMB(Red) (0.020 mol/l) was prepared in double-distilled water and kept in a dark bottle. The substrate solution consisted of 2.0×10^{-4} mol/l TMB(Red), 1.0×10^{-2} mol/l Na_2HPO_4 and 5.0×10^{-3} mol/l citric acid (pH 5.0). The running buffer consisted of 2.0×10^{-3} mol/l H_2O_2, 2.5×10^{-4} mol/l $Na_2B_4O_7$ and 9.0×10^{-3} mol/l H_3BO_3 (pH 7.4). TMB(Red) or H_2O_2 was added to the buffers just before the measurement. The running buffer was renewed every run. All buffers and solutions were stored at $4\,°C$ until use. Unless stated otherwise, all other reagents were of analytical grade or better and purchased from standard reagent suppliers. All solutions were prepared with double-distilled water. All buffers were filtered through 0.45-μm cellulose acetate membrane filters (Shanghai Yadong Resin Co. Ltd., Shanghai, China) before use.

2.3. Immunoassay procedure

The immunoassay protocol was a noncompetitive format. A 25-μl aliquot of the CA15-3 standards or serum samples, and a 5-μl aliquot of HRP-labeled anti-CA15-3 antibody were added to a microcentrifuge tube. The solution was incubated for 1 h at room temperature, and then was diluted to 150 μl with the running buffer. Before injection, the levels of the solutions in the sample vial (not shown in Fig. 1), the running buffer reservoir (2) and the catalysis reactor (5) were kept at the same height. The liquid pressure buffer reservoir (3) and the liquid pressure substrate reservoir (4) were put 40 cm over the running buffer reservoir and the catalysis reactor, respectively. The injection process was as follows: first, the liquid pressure substrate reservoir (4) was put down and keeps the solution level at the same height as that of the catalysis reactor (5). The switch (8′) of the liquid pressure substrate reservoir (4) was turned on. The switch (8) of the liquid pressure buffer reservoir (3) was turned off. Then hydrodynamic injection was carried out by inserting the inlet of the separation capillary into the sample vial

and raising the vial 9 cm height for 20 s. After injection, the separation capillary was manipulated down, out of the sample vial, and then immersed in the running buffer solution. After that, the cover (9) of the running buffer reservoir was sealed. The switch (8) of liquid pressure buffer reservoir was turned on, and then the liquid pressure substrate reservoir was raised 40 cm. Finally, the separation high voltage was applied across the separation capillary, the detection potential was applied at the working electrode and the electropherogram was recorded. During the electrophoresis, the same liquid pressure from the liquid pressure buffer reservoir and the liquid pressure substrate reservoir was kept to prevent a distortion of the flat electroosmotic flow profile in the separation capillary. The reaction time could be controlled by the liquid pressure. Routinely, for a 50 μm I.D. reaction capillary, the applied liquid pressure height was 40 cm.

In the electrochemical detection, the working microdisk bundle electrode was cemented onto a microscope slide, which was placed over a laboratory-made XYZ micro-manipulator and glued in place in such a way that the microdisk end protruded from the edge of the slide. The position of the microdisk bundle electrode was adjusted (under a microscope) against the outlet of the reaction capillary so that the electrode and the capillary were in contact. This arrangement allowed easy removal and realignment of both the capillary and the electrode. All potentials were measured against SCE. All disposable plastic wares and disposable micropipette tips used in the assay were autoclaved prior to use in order to denature any contaminants. All solutions were prepared in disposable plastic ware using disposable pipette tips.

3. Results and discussion

3.1. Optimization of CE-EIA-ED

The electropherograms of HRP-labeled anti-CA15-3 antibody (Ab*) in different buffers of pH 7.4 are shown in Fig. 2. It can be found that the maximum peak area, q, the minimum width at the half-peak, $W_{1/2}$ and the maximum number of theoretical plates, N, were obtained in 9.0×10^{-3} mol/l
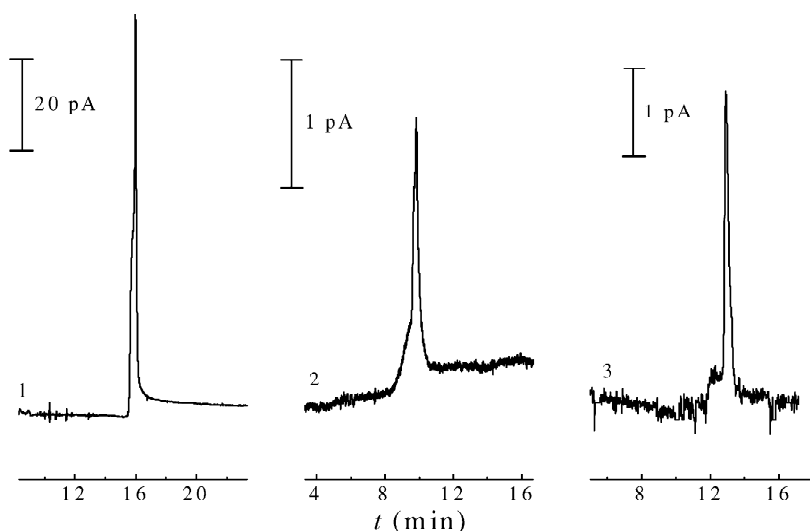
Fig. 2. Electropherograms of HRP-labeled anti-CA15-3 antibody, Ab*, in: (1) 9.0×10^{-3} mol/l Na_2HPO_4–4.6×10^{-4} mol/l citric acid; (2) 1.0×10^{-2} mol/l Tris–8.4×10^{-3} mol/l HCl and (3) 9.0×10^{-3} mol/l H_3BO_3–2.5×10^{-4} mol/l $Na_2B_4O_7$; 1.67 mg/l Ab* and 2.0×10^{-3} mol/l H_2O_2 in the running buffer; substrate solution, 2.0×10^{-4} mol/l TMB(Red)+1.0×10^{-2} mol/l Na_2HPO_4–5.0×10^{-3} mol/l citric acid (pH 5.0); separation capillary, 25 cm×50 μm I.D., reaction capillary, 5 cm×50 μm I.D.; h, 40 cm; hydrodynamic injection, 9 cm for 20 s; separation voltage, 20 kV; detection potential, 0.00 V.

H_3BO_3–2.5×10^{-4} mol/l $Na_2B_4O_7$ (pH 7.4). If the buffer concentration is higher than this value or pH>7.4, q will decrease. Therefore, this buffer was selected in the subsequent experiments.

The migration time, t_m, q, $W_{1/2}$, and N at different V_s are listed in Table 1. t_m and $W_{1/2}$ decrease and N increases very slowly with increasing V_s. When $V_s <$ 20 kV, q is a constant. When $V_s >$ 20 kV, q decreases. Therefore, 20 kV for V_s was chosen in our experiments. Fig. 3 shows the relationship between q and the applied detected potential, E_d. When E_d is higher than 0.10 V, q increases with decreasing E_d. When E_d is lower than 0.10 V, q is almost a constant. E_d of 0.00 V was used because of larger q and lower noise.

The liquid heights, h, in the system shown in Fig. 1 obviously exerted an influence on q. When $h <$ 40 cm, q increases rapidly with increasing h. When $h >$ 40 cm, q decreases with increasing h. q has a maximum at the liquid height of 40 cm. t_m decreases slowly with increasing h. N and $W_{1/2}$ are almost constants with increasing h. Therefore, 40 cm was selected in our experiments. It was found that $2.0 \times$
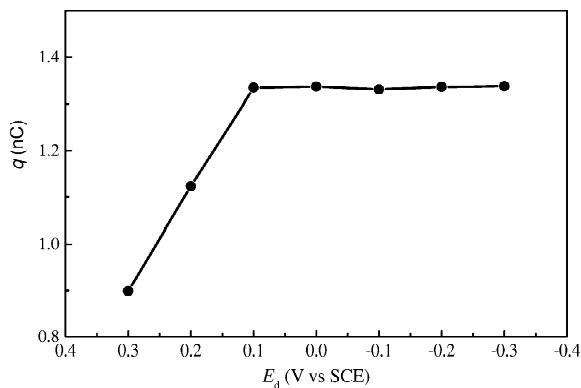
Table 1
The values of q, t_m, $W_{1/2}$ and N at different separation voltages, V_s

V_s (kV)	t_m (min)	q (nC)	$W_{1/2}$ (s)	$10^{-4} N$
12	16.6	1.30	10.6	4.9
15	16.3	1.31	10.0	5.3
18	16.2	1.31	9.9	5.3
20	16.1	1.34	9.8	5.4
25	15.9	1.11	9.6	5.4

Conditions: 9.0×10^{-3} mol/l H_3BO_3–2.5×10^{-4} mol/l $Na_2B_4O_7$, pH 7.4; other conditions as in Fig. 2.

Fig. 3. Relationship between the detected electric charge, q, and the applied detected potential, E_d. 9.0×10^{-3} mol/l H_3BO_3–2.5×10^{-4} mol/l $Na_2B_4O_7$ (pH 7.4). Other conditions as in Fig. 2.

10^{-4} mol/l TMB(Red) and 2.0×10^{-3} mol/l H_2O_2 were optimum in our experiments.

3.2. CE-EIA-ED for CA15-3

In this method, the noncompetitive format was carried out. CA15-3 (Ag) reacted with an excess amount of HRP-labeled anti-CA15-3 antibody (Ab*). After the noncompetitive reaction was completed, the solution contained Ab* and the complex (Ag–Ab*). Both were separated by CE in the separation capillary. Both could catalyze TMB(Red) (from the liquid pressure substrate reservoir) and H_2O_2 (flowing out from the separation capillary with the running buffer) in the reaction capillary following the separation capillary. The reaction product, TMB(Ox), could be detected at the outlet of the reaction capillary on the carbon fiber microdisk bundle electrode. Thus, two peaks corresponding to Ab* and Ag–Ab* should appear in the electropherograms. Under the conditions mentioned above, the electropherograms obtained are shown in Fig. 4 at different concentrations of CA15-3. With increasing the concentration of CA15-3, q of peak 1 decreases and q of peak 2 increases. According to the principle of the noncompetitive assay, peak 1 and peak 2 should be the peak of Ab* and the peak of Ag–Ab*, respectively. The peak 2 can be used for quantification of CA15-3. The calibration curve based on the peak of Ag–Ab* is shown in Fig. 5. The points represent the average q detected for three runs. The limit of detection (LOD) for the noncompetitive assay calculated using the mean q of the Ag–Ab* peak for the zero-dose CA15-3 plus three times its standard deviation calculated from 10 trials was 0.024 U/ml, which is lower than the value (0.3 U/ml) obtained by immunoradiometric assay [22]. According to the Hagen–Poiseuille equation, the injection volume calculated was 5.5 nl for the injection with 6.0 cm height for 20 s. Therefore, a mass LOD of 1.3×10^{-7} U can be obtained. The response for a series of eight injections of the solution containing 20.8 U/ml CA15-3 resulted in a relative standard deviation of 6.3% for q and 4.4% for t_m. In order to verify the method, two serum samples from different breast tumor patients were detected according to the procedure described in Section 2. In order to make the concentrations of the
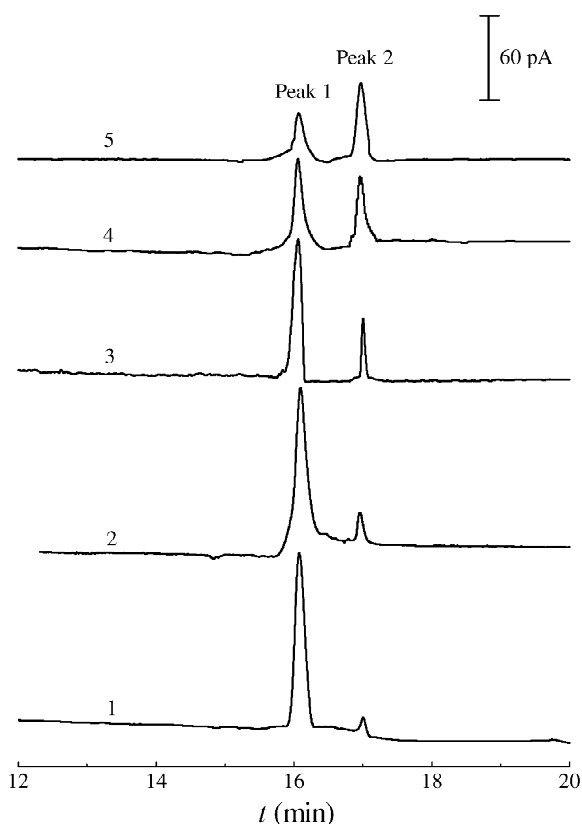

Fig. 4. Electropherograms of the solutions for different concentrations of CA15-3. Concentration of CA15-3 (U/ml): 1, 0; 2, 2.50; 3, 8.33; 4, 20.8; 5, 41.7; 9.0×10^{-3} mol/l H_3BO_3–2.5×10^{-4} mol/l $Na_2B_4O_7$ (pH 7.4). Other conditions as in Fig. 2.
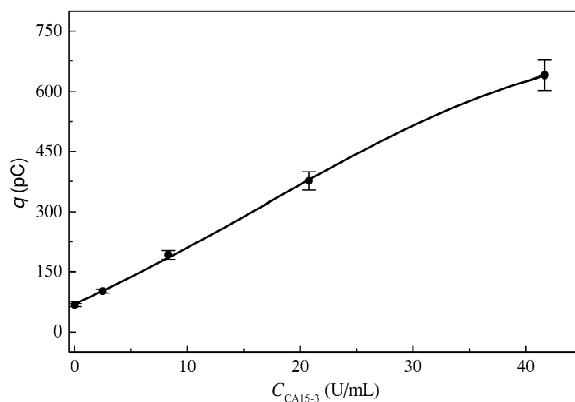

Fig. 5. Calibration curve based on the peak of the complex of CA15-3 with its antibody, Ag–Ab*; 9.0×10^{-3} mol/l H_3BO_3–2.5×10^{-4} mol/l $Na_2B_4O_7$ (pH 7.4). Other conditions as in Fig. 2.

Table 2
Concentrations of CA15-3 detected and recovery in the diluted serum samples

Sample	Determined value (U/ml)	Average value (U/ml)	Added value (U/ml)	Observed value (U/ml)	Recovery (%)
I	11.4		6.67	18.0	99
	11.2	11.3	8.33	19.2	96
	11.2		10.0	21.5	103
II	8.75		6.67	15.0	94
	8.57	8.76	8.33	16.3	93
	8.97		10.0	18.7	97

Conditions: 9.0×10^{-3} mol/l H_3BO_3–2.5×10^{-4} mol/l $Na_2B_4O_7$, pH 7.4; other conditions as in Fig. 2.

sample solution detected in the linear part of the calibration curve, the serum samples were diluted before analysis. The results for the diluted samples are shown in Table 2. The concentrations of CA15-3 in the two samples obtained by standard addition are 67.8 and 52.6 U/ml, respectively, which agreed with the values (69.3 and 50.0 U/ml) determined by ELISA. When the standard CA15-3 was added into the sample, the determined recovery was between 93 and 103%.

4. Conclusions

CE-EIA-ED with the noncompetitive format developed is a new useful method with high selectivity, low LOD and low sample consumption for biological substances. In many commercially available enzyme immunoassay kits, HRP is labeled on antigen or antibody, and the TMB(Red) is used as enzyme substrate. Therefore, CE-EIA-ED with a noncompetitive format based on the catalysis action of HRP can easily be used to determine other analytes. This method is useful where the HRP enzyme label is available and a fluorescent label is not. We think that CE-EIA-ED will become a useful tool in immunological assays.

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