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# A sensitive immunoassay for determination of hepatitis B surface antigen and antibody in human serum using capillary electrophoresis with chemiluminescence detection

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**Abstract**

A sensitive and homogeneous immunoassay (IA) based on capillary electrophoresis (CE) with enhanced chemiluminescence (CL) detection has been developed for the determination of hepatitis B surface antigen (HBsAg) and antibody (HBsAb) in human serum. The conditions for the CL reaction and electrophoresis were investigated in detail using horseradish peroxidase (HRP) labeled HBsAg (HBsAg*) as a marker because of its catalytic effects on the luminol–hydrogen peroxide reaction. The CL reaction was enhanced by para-iodophenol and the CL detector was designed uniquely without any dead volume or diluents effect. The present method has been used for assaying HBsAg and HBsAb in human serum using a competitive format and a non-competitive format, respectively. Under the optimal conditions, the linear ranges were from 1 to 400 pmol/L ($R = 0.9988$) for HBsAg and 2 to 200 mIU/mL ($R = 0.9981$) for HBsAb. The detection limits were 0.4 pmol/L and 1 mIU/mL for HBsAg and HBsAb, respectively. The relative standard deviations of peak area were 4.2% and the errors of it were from −0.03% to +0.05% for 80 pmol/L HBsAg* ($n = 7$). In this study, the free HBsAg* and the bound HBsAg* (HBsAg*–HBsAb) were separated in the separation capillary within 6 min using a borate run buffer. To verify the experimental reliability, the result was comparable with that of enzyme linked immunosorbent assay (ELISA) and demonstrated the feasibility of the CE–CL immunoassay method for clinical diagnosis.


*Keywords:* Immunoassay; HBsAg; HBsAb; Capillary electrophoresis; Chemiluminescence

## 1. Introduction

The human B hepatitisis is one of the most widespread infectious diseases and it can induce chronic or acute hepatitis. There are approximately 300 million people that are still suffering from this detrimental or fatal disease around the world. In China alone, about 60% of above-mentioned people have been infected with hepatitis B or about 10% of Chinese people have been infected with it [1]. It is generally accepted that the diagnosis of infection by hepatitis B virus (HBV) is based on the presence of the HBsAg in the blood, since that it can generally be detected while still in the incubation period [2]. To patients, HBsAb is the symbol for restoring to health after infecting with HBV and to healthy people, injecting the hepatitis B bacteria, as successful immunoreactions should generate HBsAb. On the fast development of the clinical diagnostic technologies, there were some case reports on detection of HBV DNA by polymerase chain reaction (PCR) technology of molecular biological methods [3,4] as well as the chip-based detection method [5]. Currently, surface plasmon resonance biosensor [6] and immunovoltammetry with nano-magnetic microsphere [7] technologies were reported, while enzyme-linked immunosorbent assay (ELISA) was still the main means to clinically diagnose HBV [8,9]. However, the shortcomings of ELISA method are indirect, long analytical time and low sensitivity. Therefore, it is necessary to develop a simple, rapid, and effective method to detect HBV in real samples.

Compared to conventional ELISA and radioimmunoassay (RIA) [10], CE combined with IA is characterized by high resolution and less sample, and will become a powerful tool in clinical diagnosis, environmental and food analyses. There were detailed reviews of CEIA in 1997, 2000 and 2003 [11–13].

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High-performance liquid chromatography (HPLC) is the regular means in comparison with ELISA due to its broad application in pharmaceutical analysis and clinical diagnosis [14–18], while its applicability in IA including sensitivity isn't comparable to that of CEIA. Therefore, no reports were found using HPLC methods for determination of HBV. Laser induced fluorescence (LIF) detection is still the usual choice in CEIA because of its high sensitivity, commercially available apparatus and the ease in the preparation of fluorescent-labeled tracer [19–27]. Apart from LIF detection, CL detection has received much attention due to its high sensitivity, low cost, wide linear range and having no use for excitation sources, while few reports have been found using CEIA with CL detection [28,29] including the successful application of it to quantification of CA125 in human sera and determination of bone morphogenic protein-2 in rat vascular smooth muscle cells.

In the present paper, a sensitive and homogeneous IA based on CE–CL detection has been developed for the determination of HBsAg and HBsAb in human serum. The conditions for the CL reaction and electrophoresis were investigated in detail using HBsAg* as a marker because of its catalytic effects on the luminol–hydrogen peroxide reaction with the para-iodophenol as a CL enhancer. Since this CL reaction is a fast and weakly luminescence reaction, it is necessary that the location of this CL reaction should be controlled accurately at the detection windows. The zone of enzymatic marker which was discharged from the end of the separation capillary and at the center of the detection window could be reacted immediately with the CL reagents, which were transported continuously in the reaction capillary. Thus a unique CL detector was designed so extraordinary that it can obtain the most luminescent intensity. The HBsAg* reacted with a limited amount of HBsAb, and then formed a non-competitive CEIA. The determined HBsAg was added to above fixed mixture and thus unlabeled HBsAg competed with HBsAg* for binding to a limiting amount of HBsAb, and this was a competitive CEIA. Separation of the mixture by CE with CL detection produced two distinct peaks corresponding to HBsAg* and HBsAg*–HBsAb complex, the intensities of which can be related to the original concentration of HBsAg or HBsAb. In CE separations performed in fused-silica capillaries coated with polyimide, the adsorption of the capillary significantly affected the separation efficiencies of proteins. Therefore, the use of high-pH buffer was adopted for suppression protein adsorption [30], in which the proteins were changed into negatively charged species.

The present method has been employed to determination of the HBsAg and HBsAb in human serum. The result was comparable with that of enzyme linked immunosorbent assay (ELISA) and in CE–CL, the sensitivity was higher than that of ELISA method [31]. Its detection limit is 0.4 pmol/L for HBsAg and 1 mIU/mL for HBsAb, and this value is also lower than that of time resolved fluoroimmunoassay with 0.2 ng/mL for HBsAg and 5 mIU/mL for HBsAb [32]. The relative standard deviations of peak area were 4.2% and the errors of it were from −0.03% to +0.05% for 80 pmol/L HBsAg* ($n = 7$). Thereby, this method demonstrated the feasibility of the CE–CL immunoassay method for clinical diagnosis. In the future, the analytical system of CE–IA based on CL detection can be integrated into an array capillary electrophoresis chip and thus, a large number of samples could be synchronously determined along with overall improved analytical efficiency.

## 2. Materials and methods

### 2.1. Apparatus

A MPI-A CE–CL detection system including a 0–20 kV high voltage (HV) power supply and analytical system of multimember data collection (Ruimai electronic technological corporation, Xi'an, China) was used throughout the experiment (Fig. 1A). A fused-silica capillary (50 cm × 75 μm I.D.) coated with polyimide (Polymicro Technologies, Phoenix, AZ, USA) was used for separation, and then inserted into a reaction capillary (18 cm × 620 μm I.D.; Chongqing Optical Fiber, Chongqing, China). The detection window was formed by burning 8 mm of the polyimide of the reaction capillary and setting it in front of the photomultiplier tube (PMT; Hamamatsu, Japan), and synchronously putting a reflector on the other side. A section of the end of the separation capillary was inserted in the middle of the detection window (Fig. 1B). The CL reaction proceeds immediately in the detection window and there is no dead volume or diluents effect in the reaction capillary in such elaborate device. The distance between the reaction capillary detection window and the PMT was 3 mm. The PMT of the detector was operated at 700–800 V. CL reagents were delivered by a double microsyringe pump (Shanghai Instrument Plant, Shanghai, China) and flowed through a reagent capillary (22 cm × 250 μm I.D.; Lanzhou Institute of Chemical Physics, China) to the reaction capillary. CL reagent solutions were fed at a rate of 15–20 μL/min. The end section of the reaction capil-

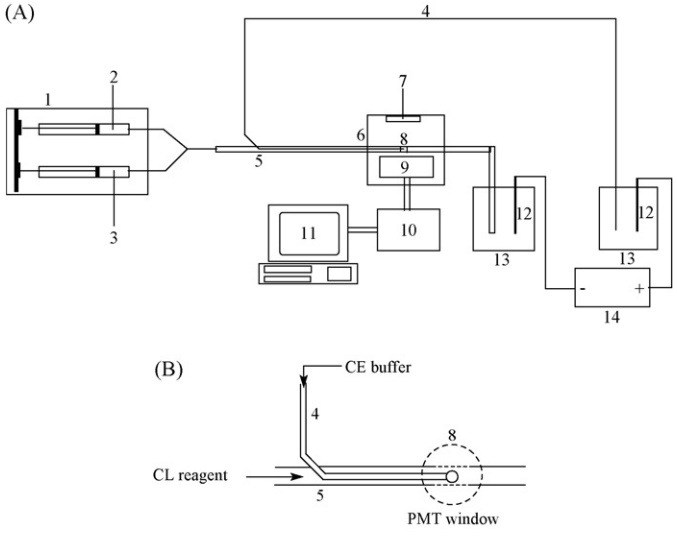


Fig. 1. Schematic diagram of the capillary electrophoresis instrument with chemiluminescence detection. (A) (1) double syringe pumps; (2) luminol solution; (3) $H_2O_2$ solution; (4) electrophoresis separation capillary; (5) reaction capillary; (6) black box; (7) reflector; (8) PMT window; (9) PMT; (10) signal amplifier; (11) personal computer; (12) Pt electrodes; (13) electrolyte reservoirs; (14) high-voltage power. (B) Schematic of CL detection interface.

lary exited the detector and entered a buffer reservoir to complete the circuit. Data acquisition and collection were processed using commercially available software (IFFM–D data analysis system, Xi'an, China). The pH of buffer solutions was measured by a precise pH meter (pHs-2, Shanghai Second Analytical Instrument Factory, Shanghai, China).

### 2.2. Chemicals (reagents and solutions)

Luminol was from Merck (Darmstadt, Germany). Hydrogen peroxide (30% $H_2O_2$), acetic acid, sodium hydrogen carbonate ($NaHCO_3$), sodium hydroxide (NaOH), ethylene diamine tetraacetic acid (EDTA), and sodium tetraborate ($Na_2B_4O_7$) were purchased from Shanghai Chemical Plant (Shanghai, China). Horseradish peroxidase (HRP) was obtained from Boaosen Biochemcals (Beijing, China). Para-iodophenol (PIP) was a product from J&K (Shanghai, China). The HBV ELISA kit (T 2000189), which contained HRP-labeled HBsAg, standard HBsAb and HBsAg and all of them have already prepared by spiked human serum were purchased from Kehua Biochemical (Shanghai, China). The electrophoresis buffer was 4.0 mmol/L $Na_2B_4O_7$ (pH 10.20), and the CL reaction buffer was 50 mmol/L $NaHCO_3$ (pH 9.5) containing $8.0 \times 10^{-3}$ mol/L $H_2O_2$, $7.0 \times 10^{-4}$ mol/L luminol, $1.0 \times 10^{-4}$ mol/L EDTA and $6.0 \times 10^{-4}$ mol/L PIP. Sodium phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate, 138 mmol/L NaCl, 27 mmol/L KCl, pH 7.4) was used as diluents. All solutions were prepared with deionised water obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA) and filtered through 0.22 μm membrane filters prior to use. Human serum samples were provided by Southwest University Hospital and kept at −20 °C.

### 2.3. Preparation of capillaries

The new capillary was initially rinsed with 1 mol/L NaOH for half an hour, followed by 0.1 mol/L NaOH for 20 min, then with Milli-Q water for 20 min, and finally with the buffer solution for 20 min. To maintain reproducible migration times, the capillary was flushed with 0.1 mol/L NaOH for 2 min, water for 2 min, running buffer for 3 min between runs. The sample was introduced by electrokinetic injection with a voltage of 10 kV for 8 s, and 15 kV was applied to separation for 500 s with current reading of about 20 μA. The CL reaction and running buffer were refreshed every 2 h. In order to keep the capillary wall in good condition, 0.1 mol/L NaOH was filled in it when not in use.

### 2.4. CL conditions

Combined with the above procedure, the double microsyringe pumps were switched on to provide a mixed constant flow of CL reagent (luminol–$H_2O_2$ solution) to the reaction capillary during analysis. The rate of the double microsyringe pumps was 10 μL/min with the 5 mL of the microsyringe volume. The reaction capillary was also washed by 0.5 mol/L NaOH after use for a day, and rinsed with water and CL reagent before use.

### 2.5. IA procedure

To determine the HBsAb, a non-competitive format was adopted and indicated as following:

$$\mathrm{Ab} + \mathrm{Ag}^* = \mathrm{Ag}^* + \mathrm{Ag}^*\text{–}\mathrm{Ab} \quad (1)$$

where Ab is the HBsAb with a limited amount, and Ag* is the HBsAg* with an excessive fixed amount. Although the analyte (Ab) is not directly detected, its concentration is related to the amount of Ag* and Ag*–Ab in the system. Therefore, the response of Ag* and Ag*–Ab can be used to quantify the concentration of Ab.

There is a direct proportion of the concentration of Ab solution to the CL intensity of Ag*–Ab.

If an increasing amount of unlabeled Ag was added to above fixed mixture and thus unlabeled Ag competed with Ag* for binding to a limiting amount of Ab, and formed a competitive format. Thus, Ag could be determined based on above state and the principle was also illuminated using the formula:

$$\mathrm{Ag} + \mathrm{Ag}^* + \mathrm{Ab} = \mathrm{Ag} + \mathrm{Ag}^* + \mathrm{Ag}\text{–}\mathrm{Ab} + \mathrm{Ag}^*\text{–}\mathrm{Ab} \quad (2)$$

It is known that Ag and Ag-Ab couldn't be observed because of absent HRP tag. So the formula can be predigested:

$$\mathrm{Ag} + \mathrm{Ag}^* + \mathrm{Ab} = \mathrm{Ag}^* + \mathrm{Ag}^*\text{–}\mathrm{Ab} \quad (3)$$

As the concentration of unlabeled Ag increased, the peak height and area of the immuno-complex Ag*–Ab in the electropherogram decreased while the free Ag* increased. There is a direct proportion of the concentration of Ag solution to the CL intensity of Ag*.

Ag*, Ag, and Ab spiked human serum were diluted to the appropriate concentrations with 10 mmol/L PBS, pH 7.4. To perform non-competitive assay, a 50 μL volume each of fixed Ag* and different Ab solution were mixed in a 500 μL microcentrifuge tube and incubated at 37 °C for 40 min before injection for CE–CL assay of Ab. To perform competitive assay, 50 μL of a fixed amount of Ag* was mixed with 50 μL of varying amounts of determined Ag. To each was added 50 μL of fixed amount of monoclonal antibody. These mixtures were allowed to incubate at 37 °C for 40 min and were analyzed by CE.

## 3. Results and discussion

### 3.1. Optimization of CL conditions

As the chemiluminescence reagent, luminol and $H_2O_2$ concentrations affect the CL intensity. We examined the effects of the concentrations of luminol and $H_2O_2$, CL reaction buffer solution and flow rate of double microsyringe pump on CL intensity in presence of HBsAg* with a concentration of 100 pmol/L. The CL intensity reached the maximum value at the $H_2O_2$ and luminol concentrations of $8.0 \times 10^{-3}$, $7.0 \times 10^{-4}$ mol/L, respectively. We also investigated several CL reaction buffer

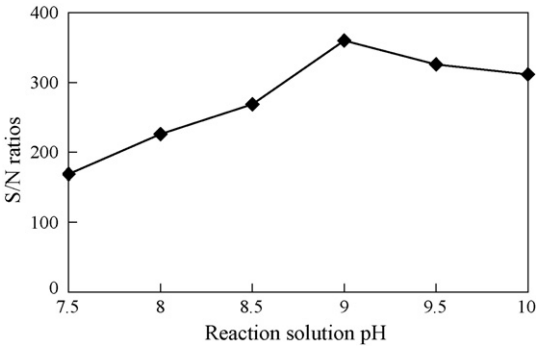


Fig. 2. pH effects of the CL reactions solutions in the presence of 100 pmol/L HBsAg*. $NaHCO_3$ solution (50 mmol/L, pH from 7.5 to 10) containing $8.0 \times 10^{-3}$ mol/L $H_2O_2$, $7.0 \times 10^{-4}$ mol/L luminol, $6.0 \times 10^{-4}$ mol/L PIP, and $1.0 \times 10^{-4}$ mol/L EDTA was used as a reaction buffer. Sodium borate (4.0 mmol/L, pH 10.2) was used as a run buffer. Applied separation voltage 15 kV, sample injection time 8 s with electrokinetically introduced at 10 kV. The temperature was about 25 °C.

solutions and found that the CL emission in the $NaHCO_3$ solution was more stable than in other buffers such as PBS, NaOH, $NaHCO_3$–NaOH, $Na_2CO_3$–$NaHCO_3$, Tris–HCl, and acetate buffer. Fig. 2 shows that the CL intensity of HBsAg* rapidly increased with an increase in the $NaHCO_3$ buffer pH at low pH range, and then the CL intensity decreased below pH 9.0. So the optimum pH of the reaction solution was 9.0 in the following work, which was close to the conventional IA with CL detection [33].

Under optimal conditions, the CL emission of luminol–$H_2O_2$ system was enhanced upon addition of PIP, which is the one of the broadly used CL enhancers [34]. As seen in Fig. 3, with an increasing PIP concentration, the CL emission increased and reached a maximum value at $6.0 \times 10^{-4}$ mol/L.

To mask the heavy metal ions from the analytical-grade reagents or human serum, $1.0 \times 10^{-4}$ mol/L EDTA was added to the luminol CL buffer in the presence of $H_2O_2$.

Considering the stability and sensitivity of CL emission, the flow rates of luminol–PIP mixture and $H_2O_2$ were selected at 10 μL/min, respectively. The total flow rate of substrates through the capillary to the flow cell was 20 μL/min.

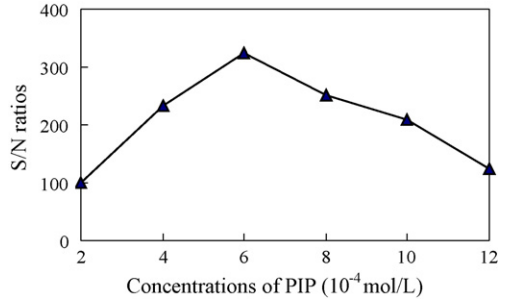


Fig. 3. Effects of PIP concentrations in the presence of 100 pmol/L HBsAg*. $NaHCO_3$ solution (50 mmol/L, pH 9.0) containing $8.0 \times 10^{-3}$ mol/L $H_2O_2$, $7.0 \times 10^{-4}$ mol/L luminol, $1.0 \times 10^{-4}$ mol/L EDTA, and PIP concentrations ranging from $2.0 \times 10^{-4}$ to $12.0 \times 10^{-4}$ mol/L. Sodium borate (4.0 mmol/L, pH 10.2) was used as a run buffer. Applied separation voltage 15 kV, sample injection time 8 s with electrokinetically introduced at 10 kV. The temperature was about 25 °C.

### 3.2. Optimization of immunoassay procedure

In an immunoassay based on the CE–CL system, analysis time is important because the complex is not stable within the long separation time. During the course of the slower run, the complex dissociates, giving no peak or poorly formed peaks for the complex. The separation time is shorter when using higher separated voltage, while it can cause higher current and unstable signal. Therefore, a voltage of 15 kV was selected for the separation. In addition, the high pH run buffer was used for suppressing the adsorption of proteins [30] in inner wall of separation capillary and gaining an appropriate EOF.

The pH of the run buffer influences the surface characteristics of the fused-silica capillary. It also plays significant roles in the conformation and charge of the HBsAg*, HBsAb and HBsAg*–HBsAb. The results showed that the conformation and charge of HBsAg* and HBsAg*–HBsAb changed greatly between pH 8.5 and 10.8. When the pH of the run buffer exceeds 11.2, the HBsAb would denature and became immunologically unresponsive and only one peak can be observed in the electropherogram. So, the optimum pH of the buffer was 10.2.

Under a given set of conditions, the buffer concentration can offer different ionic intensity and may affect resolution and migration time. When the buffer concentration was increased, the migration time and resolution of HBsAg* and HBsAg*–HBsAb increased too. When the borate buffer concentration was investigated in the range of 1.0– 8.0 mmol/L, the results showed that 4.0 mmol/L ($Na_2B_4O_7$, pH 10.2) enabled the HBsAg* and HBsAg*–HBsAb complex to be efficiently separated within 6 min along with high sensitivity.

As shown in Fig. 4, incubation time of the HBsAg* and HBsAb binding reaction (from 10 to 60 min) was optimized, and the suitable incubation time was 40 min at 37 °C.

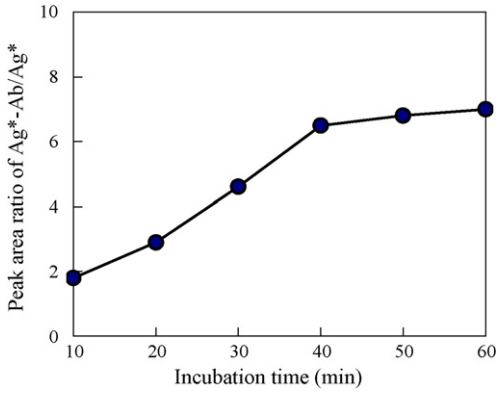


Fig. 4. Effects of incubation time. A 50 μL of 100 mIU/mL HBsAb mixed with a 50 μL of 100 pmol/L HBsAg* in a 500 μL centrifugal tube. The solution was incubated at 37 °C from 10 to 60 min. CL reaction buffer, $NaHCO_3$ solution (50 mmol/L, pH 9.0) containing $8.0 \times 10^{-3}$ mol/L $H_2O_2$, $7.0 \times 10^{-4}$ mol/L luminol, $1.0 \times 10^{-4}$ mol/L EDTA, and $6.0 \times 10^{-4}$ mol/L PIP. Sodium borate (4.0 mmol/L, pH 10.2) was used as a run buffer. Applied separation voltage 15 kV, sample injection time 8 s with electrokinetically introduced at 10 kV. The temperature was about 25 °C.

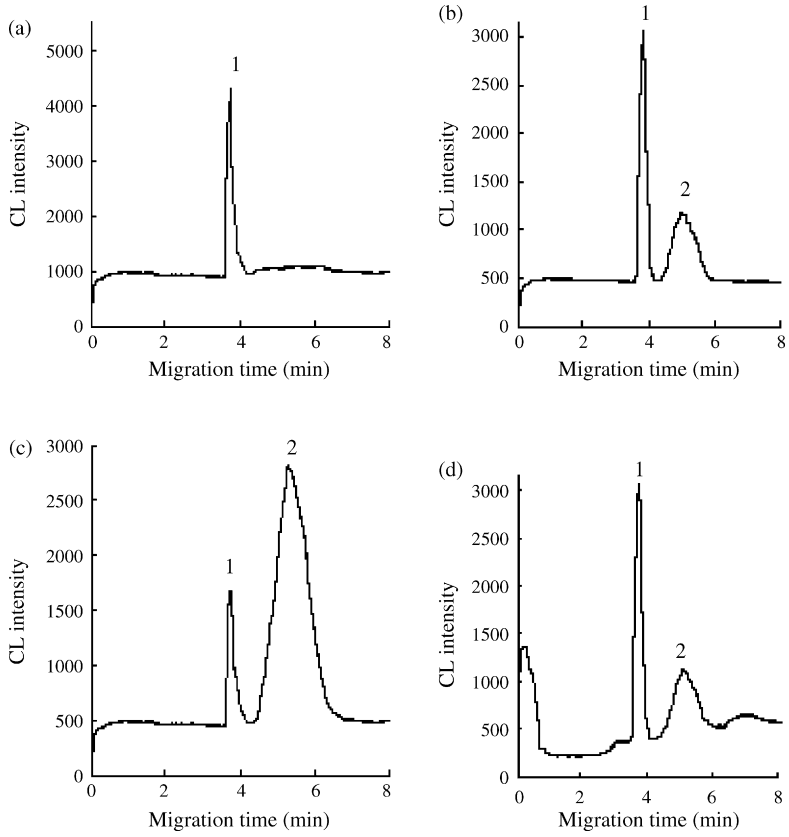


Fig. 5. Electropherograms of non-competitive immunoassay based on CE–CL of HBsAb. Each contained 500 pmol/L HBsAg*. (a) HBsAb 0 mIU/mL; (b) HBsAb 80 mIU/mL; (c) HBsAb 200 mIU/mL; (d) serum sample. Peaks: 1, HBsAg*; 2, immunocomplex. CL reaction buffer: $NaHCO_3$ solution (50 mmol/L, pH 9.0) containing $8.0 \times 10^{-3}$ mol/L $H_2O_2$, $7.0 \times 10^{-4}$ mol/L luminol, $1.0 \times 10^{-4}$ mol/L EDTA, and $6.0 \times 10^{-4}$ mol/L PIP. Sodium borate (4.0 mmol/L, pH 10.2) was used as a run buffer. Applied separation voltage 15 kV, sample injection time 8 s with electrokinetically introduced at 10 kV. The temperature was about 25 °C.

### *3.3. Determination of HBsAb in human serum using a non-competitive format*

HBsAb standard spiked human serums were diluted with 10 mmol/L PBS to the concentrations of 2, 10, 20, 40, 80, 160, 200, 400 mIU/mL. Then every 50 μL of above HBsAb solution was mixed with 50 μL of 500 pmol/L HBsAg* in a 500 μL centrifugal tube. The mixture was incubated for 40 min at 37 °C. The calibration curve was plotted through the peak areas of complex versus the concentrations of HBsAb due to a direct proportion of them. The linear range of HBsAb was 2–200 mIU/mL ($y = 0.022x + 0.76$; $R = 0.9981$) and the detection limit was 1 mIU/mL (S/N = 3 for the complex). The reaction format can be seen in formula (1).

Table 1
Results of HBsAb in human serum using a noncompetitive format

| Serum sample | ELISA* | | Diluted times | CE–CL | | |
|---|---|---|---|---|---|---|
| | OD | Qualitative results | | Peak area of Ag*–Ab | Determined (mIU/mL) | RSD% ($n = 7$) |
| 1 | 0.917 | + | 30 | 16.5 | 28 | 1.6 |
| 2 | 0.011 | − | 10 | 16 | 9 | 1.8 |
| 3 | 4.450 | + | 30 | 169 | 334 | 2.2 |
| 4 | 8.123 | + | 30 | 316 | 630 | 1.5 |
| 5 | 0.026 | − | 10 | 23.5 | 14 | 2.1 |
| 6 | 0.021 | − | 10 | 21.3 | 13 | 2.5 |
| 7 | 1.206 | + | 30 | 21.5 | 38 | 2.6 |
| 8 | 0.885 | + | 30 | 15.8 | 27 | 0.8 |
| 9 | 0.018 | − | 10 | 18.3 | 12 | 1.2 |
| 10 | 0.000 | − | 2 | 25 | 3 | 1.5 |
| Positive contrast | 1.785 | + | 30 | 28 | 61 | 1.2 |
| Negative contrast | 0.050 | − | 20 | 16 | 18 | 1.8 |

* OD is optical density and cutoff value is equal to 2.1 times OD value of negative contrast. Positive (+): sample OD value > cutoff value; negative (−): sample OD value < cutoff value.

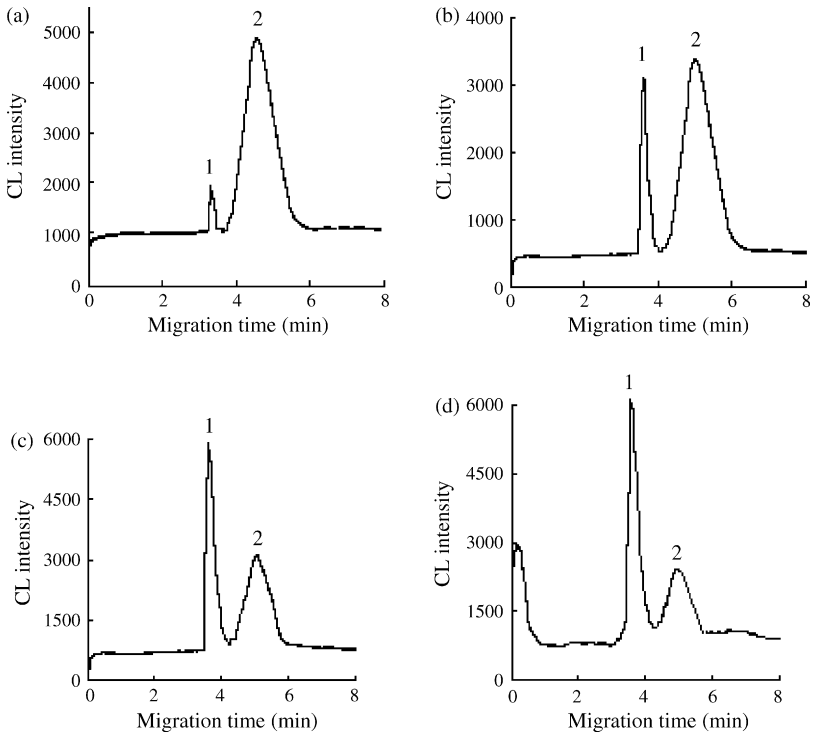


Fig. 6. Electropherograms of competitive immunoassay based on CE–CL of HBsAg. Each contained 400 pmol/L of HBsAg* and 300 mIU/mL HBsAb. (a) HBsAg 0 mol/L; (b) HBsAg 50 pmol/L; (c) 200 pmol/L; (d) serum sample. Peaks: 1, HBsAg*; 2, immunocomplex. CL and CE conditions as in Fig. 5.

Human serum samples were diluted five times with 10 mmol/L PBS for stock solution and diluted to different times again based on the CL linearity and sensitivity before determination. In the same way, 50 μL of them were mixed with 50 μL of 500 pmol/L HBsAg*, respectively and incubated for 40 min at 37 °C. The contents of HBsAb in human serum were analysed by CE–CL system as described above. Fig. 5 shows electropherograms obtained when non-competitive immunoassay based on CE was performed for the determination of HBsAb in human serum and serum spiked with HBsAb at concentrations of 42 mIU/mL. At an applied voltage of 15 kV, the free HBsAg* was separated from the complex in a run of 5.2 min. To verify the experimental reliability, the result was comparable with that of ELISA and the results of ten human serum samples can be seen in Table 1. OD is optical density and cutoff value is equal to 2.1 times OD value of negative contrast. Therefore, the positive and negative results indicated by calculating as following:

Positive (+): sample OD value >0.105; negative (−): sample OD value <0.105

When sample OD value equals to 0.105, the determined value by CE–CL equals to 22 mIU/mL. So we think HBsAb >22 mIU/mL showed positive result and HBsAb <22 mIU/mL showed negative result in human serum. The standard addition experiments were carried out according to the procedure mentioned above. The recoveries of the method were from 98 to 105%, and the RSD were less than 4% ($n = 7$).

Table 2
Results of HBsAg in human serum using a competitive format

| Serum sample | ELISA* | | Diluted times | CE–CL | | |
|---|---|---|---|---|---|---|
| | OD | Qualitative results | | Peak height of Ag* | Determined (pmol/L) | RSD% ($n = 7$) |
| 1 | 0.000 | − | 10 | 864 | 18 | 2.0 |
| 2 | 10.582 | + | 100 | 5333 | 24000 | 2.2 |
| 3 | 0.038 | − | 10 | 964 | 71 | 1.6 |
| 4 | 0.000 | − | 10 | 847 | 9.0 | 1.8 |
| 5 | 0.004 | − | 10 | 879 | 26 | 2.6 |
| 6 | 0.364 | + | 50 | 875 | 120 | 1.5 |
| 7 | 0.026 | − | 10 | 934 | 56 | 2.4 |
| 8 | 0.000 | − | 10 | 841 | 5.4 | 2.2 |
| 9 | 4.326 | + | 50 | 2636 | 2600 | 0.6 |
| 10 | 0.008 | − | 10 | 889 | 31 | 1.5 |
| Positive contrast | 1.467 | + | 50 | 1814 | 410 | 1.5 |
| Negative contrast | 0.050 | − | 10 | 902 | 38 | 1.8 |

* See Table 1.

### 3.4. Determination of HBsAg in human serum using a competitive format

Increasing amounts of 50 μL of HBsAg standard spiked human serums diluted with 10 mmol/L PBS (1, 24, 60, 150, 240, 300, 600, 1200 pmol/L) were mixed with 50 μL of 1200 pmol/L HBsAg* solution in 500 μL centrifugal tube, respectively. Each tube was added 50 μL of 900 mIU/mL HBsAb solution. These mixtures were incubated for 40 min at 37 °C. Because the peak heights of the HBsAg* were directly proportional to the concentrations of Ag in a range of 1–400 pmol/L ($y = 0.067x + 0.28$; $R = 0.9988$), the calibration curve was made by the peak heights of the HBsAg* versus the concentration of HBsAg according as formula (3) and the detection limit of HBsAg was 0.4 pmol/L (S/N = 3 for the HBsAg*).

During the reaction time, unlabeled HBsAg competed with HBsAg* for binding to a limiting amount of HBsAb, and this was a competitive CEIA. The preparation of serum stock solutions was similar to Section 3.3. Then diluted to certain concentration and mixed with HBsAg* before added HBsAb in 500 μL centrifugal tube. Lastly, the contents of HBsAg in human serum were directly determined by CE–CL system. As can be seen in Fig. 6, the electropherograms for the competitive immunoassay was presented including determination of HBsAg in human serum. In contrast with ELISA method, our CE–CL method was more sensitive. Table 2 shows the results of determination of HBsAg in 10 human serum samples by competitive immunoassay based on CE–CL system. Because the negative OD value also equal to 0.050, the critical OD value equals to 0.105 by calculating, and the determined value by CE–CL equals to 83 pmol/L. So, we think HBsAg >83 pmol/L showed positive result and HBsAb <83 pmol/L showed negative result in human serum. In order to verify this assay, the standard addition experiments were carried out according to the procedure mentioned above. The recoveries of the method were from 93 to 106%, and the RSD were less than 5% ($n = 7$).

The good reproducibility was likely due to the utilization of a high pH separation buffer. Under this condition, the proteins, such as HBsAg* and HBsAg*–HBsAb, were changed into the negatively charged species and their adsorptions were efficiently suppressed. The high sensitivity of our CE–CL system was mainly attributed to the use of an on-line CL reactor and the end of the separation capillary was directly inserted in the reaction capillary without any dead volume and exactly in the middle of the detection window with a reflector. In addition, HBsAg has a low molecular mass ($2.5 \times 10^4$) and its binding to the much heavier antibody ($1.6 \times 10^5$) lead to distinct migration speed difference between free antigen and antigen complex. Certainly, employment of an enhancer was also an important key.

## 4. Conclusions

In the present work, a method for the determination of HBsAg and HBsAb was developed in human serum using a competitive and a non-competitive immunoassay based on CE–CL system. The CL detector without any dead volume or diluents effect was designed uniquely. The quantitative results of CEIA techniques based on CL detection were in strong correlation with clinical ELISA methods and the sensitivity was higher than those of ELISA and time resolved fluoroimmunoassay methods. As compared with conventional immunoassay methods, CE–CL has some unexampled merits such as high sensitivity, no contamination, having no use for exciting sources, low cost and especially flexible design in detection cell. On the basis of experimental results, it is fully demonstrated the feasibility of the CE–CL immunoassay method for clinical diagnosis. In the prospective work, the analytical system of CE–IA based on CL detection could be integrated into an array capillary electrophoresis chip and thus, a large number of samples could be synchronously determined and the analytical efficiency was greatly enhanced.

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