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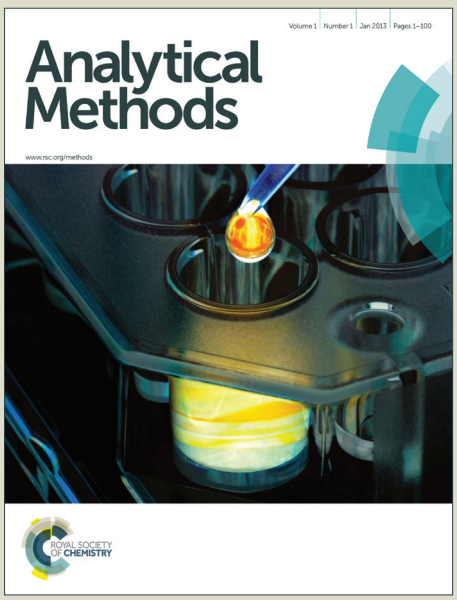

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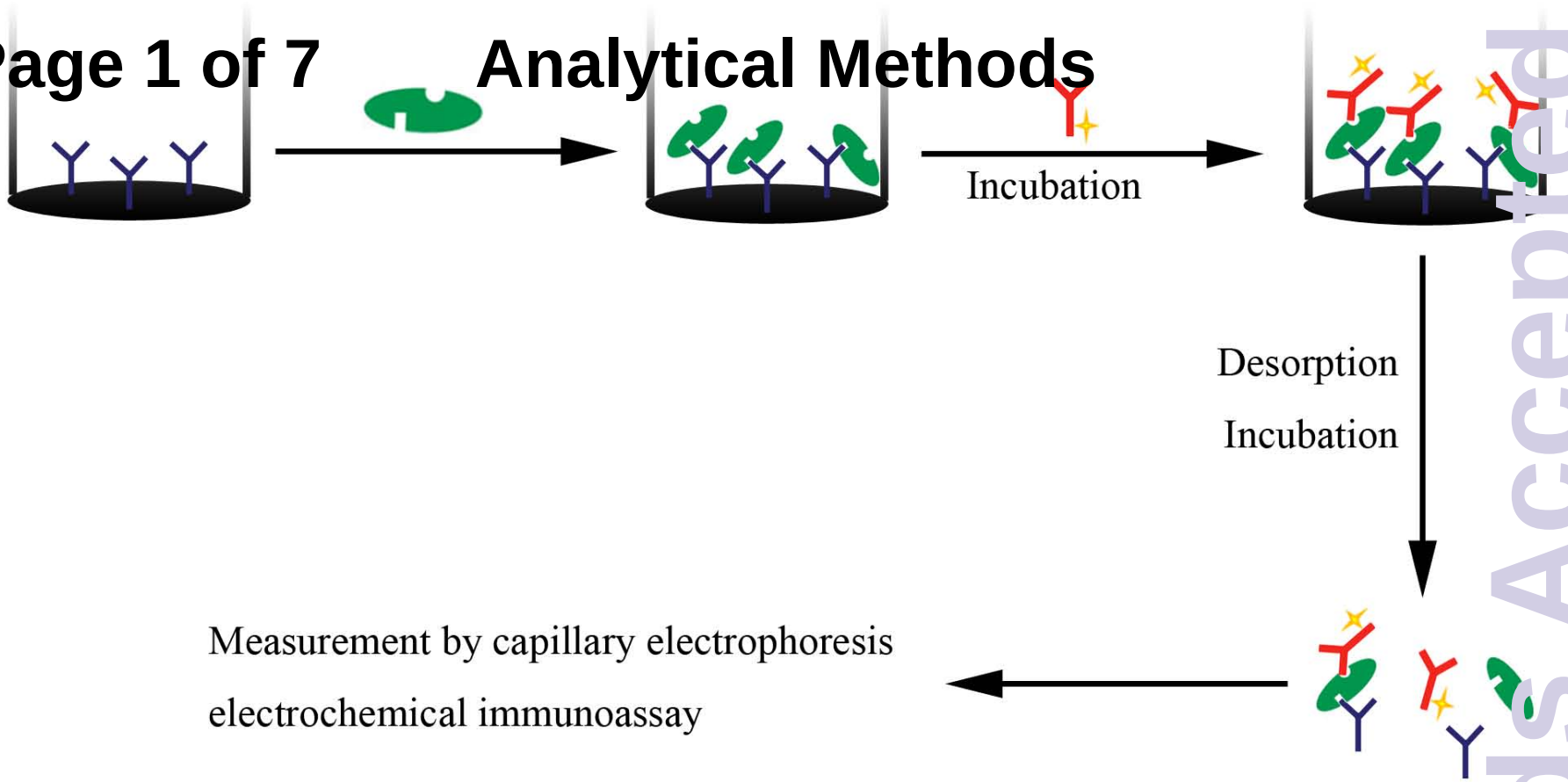

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PAPER

Determination of Hepatitis B virus surface antigen in serum with a sandwich immunoassay and capillary electrophoresis- electrochemical detection

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Sensitive and accurate detection of small quantities of hepatitis B virus surface antigen (HBsAg) is important for diagnosis of hepatitis B virus (HBV) infection and prevention of HBV transmitted disease. A novel immunoassay for determination of HBsAg in human serum was developed based on a sandwich-type protocol and capillary electrophoresis (CE) separation technology. The protocol was characterized using cyclic voltammetry (CV) and potentiostatic methods, and obtained a linear range of 0.08～10 ng ml^{-1} with a 0.01 ng ml^{-1} (3σ) detection limit. The new electrochemical immunoassay showed high sensitivity, good accuracy, high selectivity, acceptable reproducibility, fast analysis time, and better performance for determining small concentrations of HBsAg in serum compared with an enzyme-linked immunosorbent assay.

1. Introduction

Hepatitis B virus surface antigen (HBsAg) is a classic diagnostic marker for acute and chronic hepatitis B virus (HBV) infection [1, 2]. The presence of serum HBsAg indicates that an individual is probably infectious, and antigen titers correlate with the level of infection and the severity of disease [3, 4]. Patients can be infectious even if HBsAg concentrations are as low as 0.1 ng ml^{-1}. Hence, if low levels of HBsAg [5] are not efficiently detected, there is a great risk for compromised blood transfusion security and inaccurate clinical diagnoses. Consequently, sensitive and accurate detection of small quantities of HBsAg is important for the diagnosis of HBV infection and prevention of HBV transmitted disease.
Assays for quantifying HBsAg are limited. Enzyme-linked immunosorbent assays (ELISA) are currently the main assays used for detection of HBsAg, and have a detection limit of ~0.5 ng mL^{-1}. A number of novel HBsAg immunoassays with diverse formats, such as electrochemical immunoassays and fluoroimmunoassays, have recently been developed to achieve better sensitivity and accuracy and reduce the risk of false negative results. Electrochemical immunoassays have been reported to have one of four formats: (1) nanoporous gold electrodes, with horseradish peroxidase labeled secondary antibody–gold nanoparticle bioconjugates, which are about 100 times more sensitive than conventional ELISAs [6]; (2) carbon nanotube-conducting polymer networks, which was reached with 5 orders of magnitude [7]; (3) copper-enhanced gold nanoparticle used as labels and magnetic nanoparticles used as platforms for primary antibodies [8, 9]; and (4) a graphene paste electrode with gold nanoparticles and a Nafion-Lcysteine composite film was used to adsorb anti-HBs antibody, directly detected HBsAg [10]. Furthermore, fluoroimmunoassays are highly luminescent aqueous CdTe/CdS core/shell quantum dots that are covalently conjugated with anti-HBs antibody and protein G, and that provide a “flexible” coupling method to improve the sensitivity and specificity for detection HBsAg [11]. These methods, which mostly use nanomaterials or other coupling agents to improve sensitivity, are time-consuming, reagent-consuming, have high costs, and are complex.
We present a novel immunoassay that uses an efficient and specific sandwich-type protocol and sensitive capillary electrophoresis (CE) - electrochemical detection technology for HBsAg determination. Pre-coated anti-HBs monoclonal antibody (MAb), human HBsAg serum, and $Ru(bpy)_3^{2+}$-NHS ester labeled goat anti-HBs polyclonal antibody (PAb) first formed a specific sandwich-type immunocomplex on a microtiter plate, followed by desorbtion from the microtiter plate with NaOH, and finally measured using capillary electrophoresis electrochemical determination (Fig. 1). Target HBsAg was associated with $Ru(bpy)_3^{2+}$-NHS ester labeled immunocomplex, which permitted HBsAg quantification by detection of $Ru(bpy)_3^{2+}$. The test’s optimization and documentation of performance are also reported.

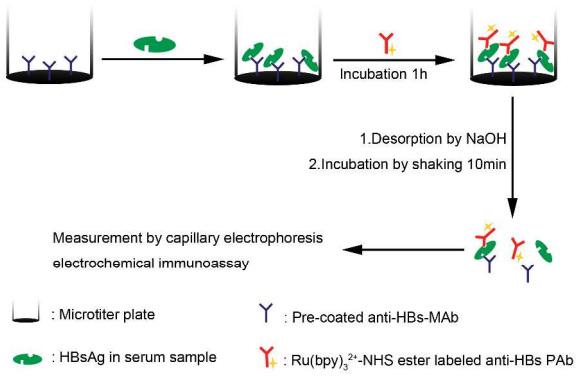

Fig. 1 Schematic illustrations of immunoassay procedures, based on a sandwich-type protocol and Capillary Electrophoresis separation for HBsAg detection.

2. Experimental

2.1. Reagents and apparatus

Bis(2,2□-bipyridine)-4,4□-dicarboxybipyridine-ruthenium di(N-succinimidyl ester) bis(hexafluorophosphate) ($Ru(bpy)_3^{2+}$-NHS ester), dimethyl sulfoxide (DMSO) and Sephadex G-25 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Tri-n-propylamine (TPA) and hydrofluoric acid were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Microtiter plates pre-coated with anti-HBs, antibody diluent, wash solution, at concentrations ranging from 0 to 180 ng mL^{-1} of HBsAg serum were obtained from Zhuhai Livzon Diagnostics Inc. (Zhuhai, China). Fetal calf serum and horse serum were purchased from Hyclone (Logan, Utah, USA). Phosphate buffer (10 mM) solutions with differing pH values were prepared by mixing 10 mM Na_2HPO_4 with 10 mM NaH_2PO_4. Clinical HBsAg serum samples (s/co values < 1.000, based on measurements using ELISA kits from Kehua Bio-Engineering Co., Ltd. (Shanghai, China)) were provided by the Clinical Laboratory of Guangzhou General Hospital of Guangzhou Command (Guangzhou, China); each serum sample was directly tested without further treatments and confirmed to be HBsAg positive and anti-HBs positive by qPCR. Ultra-pure water for the study was produced with an Aquapro water purification system(YIYANG enterprise Co., Ltd., Chongqing, China) with resistivity of 18.2MΩ cm^{-1}. All solutions were filtered through 0.22 μm Millex-GP filters (Millipore Corporation, Billerica, MA, USA) before CE analysis, and sonicated for 3 min for degassing.

A numerically controlled capillary electrophoresis (CE) high-voltage power supply (0-20 kV, Xi'an Ruimai Analytical Instruments Co., Ltd., Xi'an, China) was used to perform electrokinetic sample injection and electrophoretic separation. Separations were carried out in an open fused-silica capillary tube with an internal diameter of 100 μm, an external diameter of 365 μm, and a length of 50 cm (Yongnian ruifeng chromatographic components Co., Ltd, Hebei, China). Before first use, the 3 mm long polyimide coating on the capillary's outlet was removed with hydrofluoric acid to complete an electrophoresis loop that avoided photon absorption. The bare capillary end was subsequently cleaned with water in an ultrasonic cleaner (Pearl Electric Co., Ltd., Kunshan, China). The capillary tube was subsequently flushed with 1M HCl for 10 min, ultra-pure water for 10 min, 0.1M NaOH for 20 min, ultra-pure water for 30 min, and air-dried in N_2. Prior to each run, the capillary tube was flushed with running buffer for approximately 5 min. According to established practices [12, 13], the inner surface of capillary tubes was swept with Sodium Dodecyl Sulfate(SDS) micelles to desorb protein and subsequently filled with ultra-pure water for 5 min at the end of each run.

All electrochemical measurements were carried out on a capillary electrophoresis electrochemiluminescence detector (MPI-A) from Xi'an ruimai Analytical Instruments Co., Ltd., China. The experiments were performed with a conventional three-electrode system in a 500 μL detection cell, which was composed of a platinum disk electrode (PtE, 0.5 mm in diameter) as the working electrode, a platinum wire as the auxiliary electrode, and an Ag/AgCl (saturated KCl) reference electrode. The working electrode's surface was sequentially polished with 1 μm, 0.3 μm, and 0.05 μm alumina powders, and subsequently washed with water in the ultrasonic cleaner before use. To obtain good reproducibility, a reactivation process was done by cyclic voltammetric scanning on PtE between 0 V and 0.5 V at a scan rate of 100 mV s^{-1} for 10 cycles until the background signal was stable for electrochemical cleaning after each run [14]. The outlet of the capillary was inserted into a stainless steel tube [15], pointed to the surface of the working electrode and their distance was adjusted to about 100 μm with an inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan). Approximately 300 μL of 10 mM phosphate buffer solution was added to the detection cell for electrochemical measurements. Obstruction of the capillary was prevented with a 0.22 μm modified polyethersulfone membrane Millex-GP filter (Millipore, Billerica, MA, USA). Ultrafiltration centrifugal tubes (cut-off 30,000 MW) used to separate the unlabeled $Ru(bpy)_3^{2+}$-NHS ester were purchased from Millipore (Billerica, MA, USA). A TY-80S Shaker (Jintan Medical Instrument Factory, Jiangsu Province, China) was used for facilitating immunoreactions.

2.2. Preparation of $Ru(bpy)_3^{2+}$-NHS labeled anti-HBs PAb

A modification of an established method was used to prepare $Ru(bpy)_3^{2+}$-NHS labeled anti-HBs PAb [16]. Briefly, 1 mg of $Ru(bpy)_3^{2+}$-NHS ester was dissolved in 100 μL dimethyl sulfoxide, and then mixed with 1 ml of goat anti-HBs PAb. After 12 h of shaking at 37℃ in the dark, the resulting mixed solution was transferred to ultrafiltration centrifugal tubes and separated at 4000×g for 10 min for three

cycles to remove unlabeled $Ru(bpy)_3^{2+}$-NHS ester. Afterwards, the concentrated solution was added to a Sephadex G-25 to remove the isolated $Ru(bpy)_3^{2+}$-NHS ester by UV-visible spectroscopy and electrochemical spectroscopy. The elution was stored at 4℃ in a dark glass bottle.

2.3. Microtiter plate immunoreactions

Serum (50 µL) and 50 µL of a 1:100 dilution of $Ru(bpy)_3^{2+}$-NHS ester labeled PAb were added to each well of a polystyrene microtiter plate pre-coated with anti-HBs, and shaken for 10 min. Subsequently, the wells were incubated for 1 h at 37℃ and rinsed in washing solution six times with 1 min washing intervals. After drying, 50 µL NaOH was added to each well was added by and shaken for 10 min to desorb the $Ru(bpy)_3^{2+}$-NHS ester labeled immunocomplex. The resulting elution was stored at 4℃ prior to use.

2.4. CE and electrochemical measurement

After the pre-treated capillary and electrodes were installed into the capillary electrophoresis electrochemiluminescence detector, 400 µL of 5 mM TPA was added to the detection cell, which contained 10 mM phosphate buffer. Afterward the dark cell was closed and potentiostatic method and cyclic voltammetry (CV) measurements were performed at room temperature in the conventional electrochemical cell.

3. Results and discussion

3.1. Electrochemical behavior of the $Ru(bpy)_3^{2+}$-NHS ester labeled immunocomplex

The $Ru(bpy)_3^{2+}$-NHS ester is an electrochemiluminescence reagent which was chemiluminescent when applied to the electrode. Accordingly, the applied potential must be at least that of the analyte oxidation potential to generate the $Ru(bpy)_3^{3+}$ potential. Hence, the suitable applied potential was determined based on currents at various applied potentials. CV was used to study the electrochemical behavior. Almost no current peak can be observed for $Ru(bpy)_3^{2+}$-NHS ester labeled immunocomplex in PBS (Fig. 2a). In contrast, a pair of redox waves were clearly observed that had a potential of 1.16 V, at a scan rate of 100 mV s^{-1}, for the $Ru(bpy)_3^{2+}$-NHS ester labeled immunocomplex. This is consistent with the oxidation potential of $Ru(bpy)_3^{2+}$ and was attributed to the one-electron redox reaction of $Ru(bpy)_3^{2+}$. However, that was also the basis for choosing the initial potential for using the potentiostatic method. With a scanning voltage between 0.2 V and 0.9 V, the cyclic voltammetries of $Ru(bpy)_3^{2+}$-NHS labeled immunocomplexes and PBS were approximate, since $Ru(bpy)_3^{2+}$ was not oxidized on the electrode. In contrast, when the potential was over 0.9 V, the anodic current increased dramatically due to the electron exchange between electrochemically activated $Ru(bpy)_3^{2+}$ and radical state TPA. This enhanced electron transfer in solution.

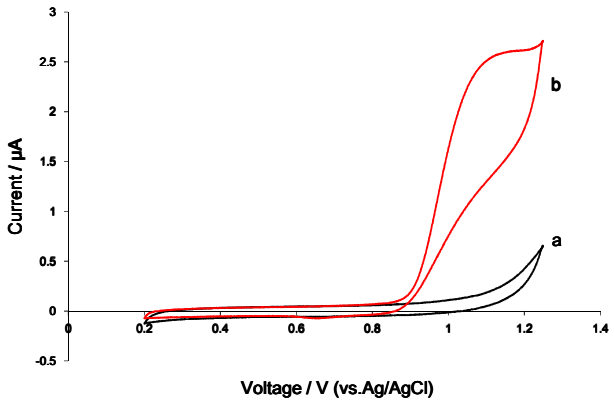

Fig. 2 Cyclic voltammograms in PBS (pH 8.0) at the bare Pt electrode. Scan rate, 100 mV s^{-1}. (a) 10 mM PBS; (b) $Ru(bpy)_3^{2+}$-NHS ester labeled immunocomplex.

3.2. Effect of NaOH concentration and desorption time

The concentration of NaOH could be used for controlling the system's pH and desorbing immune complexes from the microtiter plate. The highest peak current was achieved with 0.5M NaOH at 10 kV injection voltage, 10 s injection time, 15 kV separation voltage, 10 min elution time, 1.16 V initial potential in pH 8.0, 10 mM PBS. In addition, controlling the desorption time of NaOH was essential. If the separation time was too short, $Ru(bpy)_3^{2+}$ labeled immunocomplex did not desorb from the microtiter plate, whereas excessively long times resulted in destruction of the immunocomplex. However, desorption for 10 min resulted in the best detection results.

3.3. Effects of pH values on running buffer

One of the most important parameters in the study was the pH. This affected the electroosmotic flow, analyte charge[17], and hence, the selectivity of CE[18]. It also affected the potential window, and hence, the efficiency of Ru^{2+} electrooxidation[19].
The peak current improved with increasing running buffer pHs, up to pH 8.0, and then the peak current decreases at higher pH values (Fig. 3). This is due to increased Joule heating caused by increased ionic strength. Therefore, the optimized running buffer pH value was 8.0. This is consistent with typical optimum pH values ranging from 7-9[20], where there is good biomolecular stability and relevant electrosmotic flows.

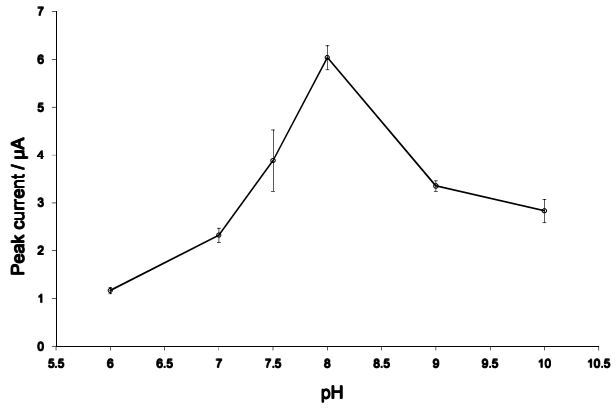

Fig. 3 Effect of running buffer pH on peak current. Conditions: sample, 180 ng mL^{-1} HBsAg; injection time, 10 s; injection voltage, 10 kV; separation voltage, 18 kV; potentiostatic method, initial potential, 1.16 V; PBS in the detection cell, pH 8.0, 10 mM.

3.4. Effect of separation voltage and injection time

The voltage applied for electrophoresis was another important parameter influencing the separation efficiency[21]. The influence of separation voltage on the peak current and migration time of the analyte was documented (Fig. 4). When the separation voltage increases, the electroosmosis flow increases, and the analyte reached the working electrode within a given time. Consequently, as peak current increases, migration time decreases at high separation voltages up to 15 kV. With separation voltages exceeding 15 kV, a lower peak current for the analyte was obtained. This may be the result of increased Joule heating in the capillary and lower concentrations of analyte at some times. Hence, a separation voltage of 15 kV was chosen with consideration for the detection sensitivity, separation performance, and migration time.

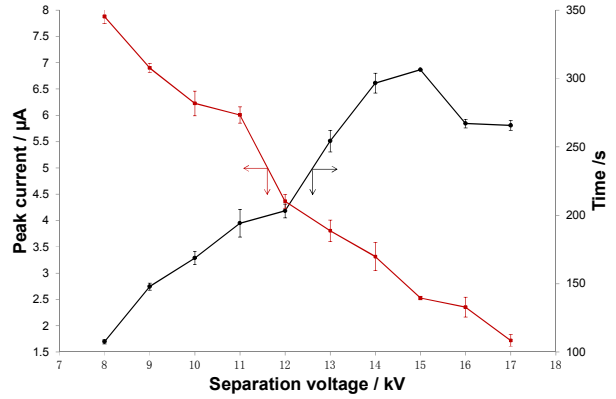

Fig. 4 Effect of separation voltage on peak current and migration time on $Ru(bpy)_3^{2+}$ labeled immune complexes. Conditions: sample, 180 ng mL^{-1} HBsAg; injection time, 10 s; injection voltage, 10 kV; potentiostatic method, initial potential, 1.16 V; PBS, pH 8.0, 10 mM.

The effect of injection time on CE separation was investigated by changing the injection time (6, 8, 10, 12 s at a voltage of 15 kV). It was found that both the peak current and peak width increased with increased injection times. However, when injection times exceed 10 s, the peak current increased slowly and the peak width increased. Therefore, 10 s (at 15 kV) was selected as the optimal electrokinetic injection time based on separation efficiencies and sensitivity.

3.5. Linearity and detection limit

Serial concentrations of HBsAg (0～180 ng mL^{-1}) were tested to determine peak current linearity under optimized experimental conditions (Fig. 5). The calibration curve for HBsAg is linear over a concentration range of 0.08 to 10 ng mL^{-1} with a regression curve of y = 0.2840 x + 0.9535 (y = peak current; x unit = ng mL^{-1}; r^2 = 0.9857). The detection limit was 10 pg mL^{-1}, and the signal-to-noise ratio was 3. This was similar to previously reported sensitivities for HBsAg electrochemical immunoassays (Table 1).

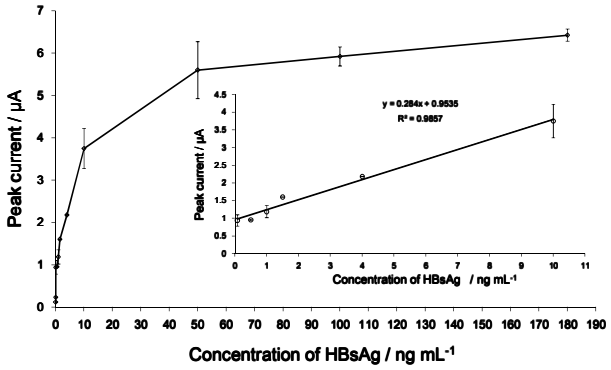

Fig. 5 Relationships between peak currents and HBsAg concentrations in serum. Conditions: injection voltage, 10 kV; injection time, 10 s; separation voltage, 15 kV; potentiostatic method, initial potential, 1.16 V; NaOH, 0.5 M; absorption time, 10 min; PBS, pH 8.0, 10 mM.

Table 1 Performance comparisons between different HBsAg electrochemical immunoassays.

Method	Technologies	Linear range	Detection limit	Ref.
Electrochemical immunoassay	using nanoporous gold electrode with HRP labeled secondary antibody–gold nanoparticles bioconjugates	0.01～1.0ng mL^{-1}	2.3 pg mL^{-1}	[6]
Electrochemical immunoassay	carbon nanotube-conducting polymer network	5 orders of magnitude	10 pg mL^{-1}	[7]
Electrochemical stripping detection	using copper-enhanced gold nanoparticle used as labels and magnetic nanoparticles used as platforms for primary antibodies	0.1～1500 ng mL^{-1}	87 pg mL^{-1}	[8]
Electrochemical detection	magnetic nanoparticles used as platforms for primary antibodies	0.001～0.015 ng mL^{-1}	0.9 pg mL^{-1}	[9]
Electrochemical detection	using the modification of a graphene paste electrode with gold nanoparticles (AuNPs) and a Nafion-Lcysteine composite film	0.5～800 ng mL^{-1}	0.1 ng mL^{-1}	[10]
The proposed method	capillary electrophoresis-electrochemical detection	0.08～10 ng mL^{-1}	10 pg mL^{-1}	

3.6. Immunoassay specificity

Specificity is one of the key considerations for a method's clinical applications. In this study, fetal calf serum and horse serum were individually analyzed with our new method. No significant changes were evident in the animal serum that we tested (Fig. 6). This indicated that our method has a high degree of selectivity for HBsAg detection.

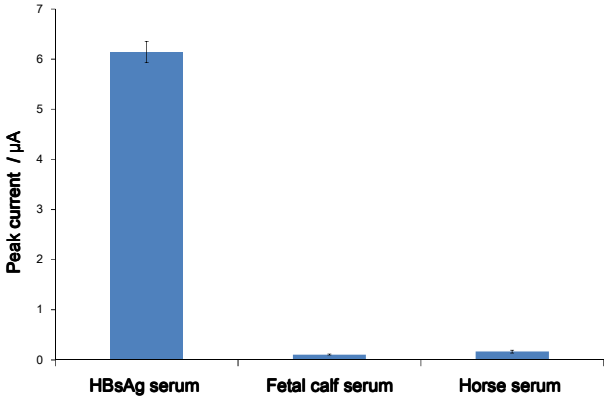

Fig. 6 Specificity of the immunoassay. The concentration of HBsAg in serum was 180 ng mL^{-1}. Fetal calf serum and horse serum were diluted ten times. Other conditions as in Fig. 5.

3.7. Sample analysis

To validate the clinical application of the proposed method, our novel electrochemical immunoassay was evaluated using electropherograms of standard HBsAg and clinical serum sample after immunoreaction. As shown in Figure 7, under optimum conditions, the migration time of both clinical and standard samples demonstrated good agreement, and no obvious interference peaks were observed in the chromatogram. These results could be attributed to the excellent electrochemical properties of $Ru(bpy)_3^{2+}$. This metal complex offers a good electron transfer channel, low background, easy labeling without affecting binding affinity, biological activity, solubility, and stability. Thus, the method showed great potential for use in electrochemical detection for bioactivators.

Furthermore, the feasibility of using our method in clinical settings was investigated by testing seven human serum samples with differing HBsAg concentrations and comparing these results with an ELISA method commonly used for HBsAg clinical determinations (Table 2). This comparison showed that our immunoassay can meet diagnostic requirements even when serum samples have small HBsAg concentrations. Our test also has better sensitivity than the conventional ELISA assay for HBsAg.

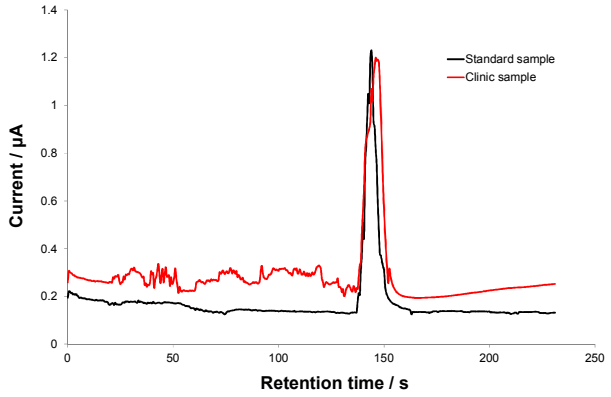

Fig. 7 Electropherogram of $Ru(bpy)_3^{2+}$ labeled immune complexes. Conditions: samples, 0.5 ng mL^{-1} standard HBsAg, clinic human serum; injection time, 10 s; injection voltage, 10 kV; potentiostatic method, initial potential, 1.16 V; PBS, pH 8.0, 10 mM.

Table 2 Comparisons of two methods for measuring human serum HBsAg levels.

Serum samples	1	2	3	4	5	6	7
The proposed method (ng mL^{-1})*	0.317	0.305	0.263	0.210	0.335	0.279	0.176
Clinic ELISA method(s/co values)	0.887	0.854	0.763	0.609	0.938	0.826	0.492

* *Average value from three successive determinations.*

4. Conclusions

A highly sensitive method for electrochemical detection of minute HBsAg concentrations, based on a sandwich-type protocol and a CE separation method was developed. The use of CE separation enhanced resolution and sensitivity of analyte detection, and also reduced analysis time and reagent consumption. Our novel CE-electrochemical immunoassay demonstrated satisfactory characteristics for HBsAg determination in human serum, such as high selectivity and a low detection limit. This study documents a simple but efficient diagnostic platform that has superior performance for clinical immunoassays for a wide variety of biomarkers, not limited to HBsAg. This will open a pathway for ultrasensitive immunoassays with broad applicability.

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Notes

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