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Diagnosis of human hepatitis B virus in non-treated blood by the bovine IgG DNA-linked carbon nanotube biosensor

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ABSTRACT

Objectives: To identify an effective bioassay for human hepatitis B virus (HBV) using voltammetric sensor.
Study design: Electrode was prepared with bovine IgG immobilized onto a DNA-linked carbon nanotube electrode (BIDCE). Analytical BIDCE conditions were optimized through square wave (SW) stripping and cyclic voltammetry (CV).
Results: Optimum parameters of 75 Hz SW frequency, 0.1 V SW amplitude, −1.3 V SW accumulation potential, 100 s accumulation time, and 3.0 mV SW increment potential were obtained. Within working ranges of 0.035–0.242 mg/mL anti-bovine IgG, the relative standard deviation of 0.2 mL HBV was observed to be 0.04 ($n = 4$).
Conclusions: Diagnostic application was performed through direct assay of HBV in non-treated human blood.

1. Introduction

Every year, more than a million people die worldwide of the hepatitis B virus[1] from human liver diseases, such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma caused by HBV.[2] Recently, various diagnostic methods have been developed for this issue, including surface plasmon resonance transducer (SPR),[3] disposable sensor techniques,[4] chemiluminescent detection,[5] VIDAS HBsAg enzyme-linked fluorescent immuno assay (ELFA),[6] MGB Taqman fluorescence PCR assay,[7] and sandwich ELISA fluorescent cytometric microbead assay.[8] Some of these methods are combined with PCR amplification, gel electrophoresis separation and photometric chemiluminescent detection methods, and which involve complicated pre-treatment techniques and expensive instruments. Moreover, such methods require photometric or other electrical detection systems. Attempts to simplify such methods have resulted in the development of other related methods, such as the electrochemical DNA sequences detection,[9] methylene blue (MB) chitosan-modified carbon paste electrode,[10] voltammetric detection in polymerase chain reaction[4] and methylene blue DNA biosensor.[11] However, these methods are dependent on PCR-amplification and height analytical detection limits. Currently, fast and inexpensive diagnostic methods are becoming in demand.[12] In this study, we first searched for a non-treated blood assay and fast diagnosis method using carbon nanotube (CNT) sensor, whose mechanical[13] and catalytic[14] properties are specific in the bioassay.[15] On its surface, DNA was linked by paste[16] and sensitively responded to the analytical target, proving its usefulness in the bio-sensing electrode. Moreover bovine IgG antigen was immobilized on the DNA-linked carbon nanotube surface. The antigen sensor was linked with the CNT–DNA–IgG···+, it can react to the antibody target with HBV, these redox voltammograms were obtained by the SW stripping and CV reaction. The analytical parameters were optimized and based on the method achieved in terms of low detection ranges for the HBV target, the developed methods can be used in direct assay of non-treated blood sera. It can also be applicable in organ monitoring, *in vivo* diagnosis, and other materials requiring HBV detection.

2. Experimental

2.1. Instrument, reagent and BIDCE preparation

Analyses were carried out using a CHI 660 A voltammetric workstation from Cordova, TN, USA. The multi-walled carbon nanotube was obtained from Nanotech Co., Ltd., Choong Nam, South Korea Zip 330-816 (by catalytic CVD, outside diameter: 15–40 nm; length: 30–50 μm). It was purified overnight prior to use by magnetic stirring in a 2 M nitric acid solution and washed using triple-distilled

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pure water. Voltammetric assay was performed using the three-electrode system. The DCE (DNA-linked carbon nanotube electrode) working electrode was linked by paste consisting of a mixture of 40 wt% carbon nanotube, 40 wt% DNA (double-stranded and prepared from calf thymus sigma) and 20 wt% reagent-grade mineral oil (New Jersey USA, 1-800-01, Acro). The mixed paste was inserted into the 3 mm diameter catheter capillary 100 mm in length, and DNA linking was performed with a 10-cycle scan, with 1.0 V initial and a −1.0 V switching potential, and a 0.5 V/s scan rate.

The BIDCE sensors were immersed in a solution of 1 mL bovine IgG for 30 min, then repeated ($n = 20$) cyclic scan was performed using 0.5 V/s scan. Thus, BI immobilization was performed in bovine IgG solution. The BIDCE can be stabilized in the electrolyte condition by cyclic scan and used for the voltammetric optimization. A 0.2 mm diameter chloride coated silver wire (as an Ag/AgCl/KCl reference electrode) and a 0.2 mm diameter platinum wire (as a counter electrode) were used. This three-electrode system was immersed in a solution of 10 mL 0.1 M $NH_4H_2PO_4$, electrolyte. Other parameters were maintained at optimal conditions. All the experiments were performed at room temperature without removing oxygen. Anti-bovine HBsAg (0.5 mg/mL), bovine IgG (8.3 mg/mL), and patient serums were obtained from the National Blood Transfusion Research Institute. The nucleotide sequences are shown as follows:

Nucleotide sequence
1 atggagagca caacatcagg attcctagga cccctgctcg tgttacaggc ggggtttttc
61 ttgttgacaa gaatcctcac aataccacag agtctagact cgtggtggac ttctctcaat
121 tttctagggg gagcacccac gtgtcctggc caaaattcgc agtccccaac ctccaatcac
181 tcaccaacct cttgtcctcc aatttgtcct ggctatcgct ggatgtgtct gcggcgtttt
241 atcatattcc tcttcatcct gctgctatgc ctcatcttct tgttggttct tctggactac
301 caaggtatgt tgcccgtttg tcctctactt ccaggaacat caactaccag cacgggacca
361 tgcaagacct gcacgattcc tgctcaagga acctctatgt ttccctcttg ttgctgtaca
421 aaaccttcgg acggaaactg cacttgtatt cccatcccat catcctgggc tttcgcaaga
481 ttcctatggg agtgggcctc agtccgtttc tcctggctca gtttactagt gccatttgtt
541 cagtggttcg tagggctttc ccccactgtt tggctttcag ttatatggat gatgtggtat
601 tgggggccaa gtctgtacaa catcttgagt ccctttttac ctctattacc aattttcttt
661 tgtctttggg tatacatttg a

Amino acid sequence
MESTTSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGG
APTCPGQNSQSPTSNHSPTSCPPICPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQG
MLPVCPLLPGTSTTSTGPCKTCTIPAQGTSMFPSCCCTKPSDGNCTCIPIPSSWAFAR
FLWEWASVRFSWLSLLVPFVQWFVGLSPTVWLSVIWMMWYWGPSLYNILSPFLPLLPI
FFCLWVYI

2.2. Property of the DCE and the BIDCE

In the electrolyte solution, the diluted 4 mL HBV patient serum was spiked, then DCE and BIDCE were compared using optimum parameters with SW stripping and CV scan. Fig. 1(A) shows the SW DCE voltammogram, in which no peak current appeared, except the −0.1 V peak current. This may indicate that −0.1 V is the DNA peak current, thus HBV signals could not be obtained. Subsequently, BIDCE was examined using the same electrolyte solution. As a result, exact and sensitive peak current was detected. Fig. 1(B) shows the −1.0 V anodic peak current, in which peak height was 0.18×10^{-6} A and a sharp peak was obtained, whose antigen and antibody electron reaction process is of (CNT–DNA–IgG$_{-}\leftrightarrow^{+}$HBV). This peak potential was used in the diagnostic assay of HBV patients. However, no cathodic peak was obtained in any of the reactions. Under such conditions, cyclic voltammetry was performed using the same electrolyte solution with 0.1 V/s scan rate, in which an identical peak potential was obtained. The results are shown in Fig. 1(C). At the positive scan, separated −1.0 V peak potential appeared, indicating that CV voltammograms can be used in HBA assay. In these conditions, reverse scan was not obtained any of the voltammograms. Moreover, in Fig. 1(C), the inserted curve was that of the CV voltammogram by electrolyte blank solution,

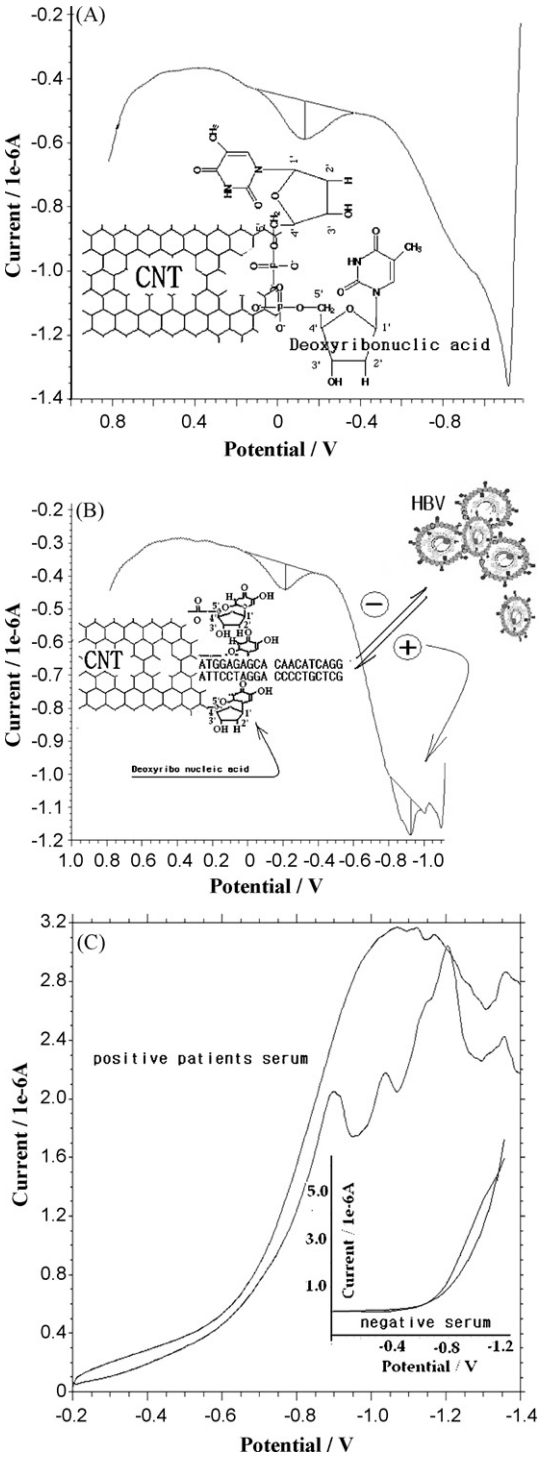

Fig. 1. (A) SW voltammograms of the DCE electrode in HBV patient serum (0.1 mL HBV patient serum was diluted in 10 mL water) 4 mL spike in 0.1 M $NH_4H_2PO_4$ 10 mL electrolyte solution. (B) BIDCE in solution (A), SW parameters of pH 4.64, SW amplitude of 0.1 V, SW frequency of 70 Hz, an increment potential of 0.03 V, an accumulation potential of −1.3 V, and an accumulation time of 300 s. (C) CV effect for BIDCE with −1.2 V initial potential, −0.2 V switching potential and 0.1 V/s scan rate in (B) solution. Insert CV is electrolyte blank solution only, and optimum conditions were set for other parameters.

which showed a linear property and from which no signal was obtained. Thus, bovine IgG immobilized effects appeared exactly, and expanded analytical properties were examined using square wave stripping voltammetry.

3. Results and discussion

3.1. SW stripping voltammetric anti-HBs and positive patient serums using BIDCE

In the 0.1 M H_3PO_4 electrolyte solution, negative serums, anti-HBs and positive patient serums were examined by BIDCE. Fig. 2(A) shows the SW comparisons. The first curve is that of a normal healthy serum of 0.1 mg/L spiked in an electrolyte blank solution, in which no signal was obtained and only a simple and linear curve appeared. In this cell, 0.1 mg/L anti-HBs was spiked and a peak height of 0.35×10^{-6} A was obtained. This was shown to be applicable for patient diagnosis by SW. Thus, patients' serums were more rigorously examined in these parameters. Fig. 2(B) shows two curves: the negative healthy serum, where no peak appeared. Subsequently, positive serums spiking appeared for −0.5 V potential at 0.27×10^{-6} A peak height, so exact and sensitive reaction current was obtained. Here, advanced SW stripping parameters were examined.

3.2. SW voltammetric optimizations of BIDCE

SW optimized conditions for the positive serums (●) and anti-HBs (○) containing the sample were examined using different cells, and the results are shown in Fig. 3. Fig. 3(A) illustrates the amplitude variations of 0.01 V, 0.03 V, 0.05 V, 0.08 V, 0.12 V, 0.18 V, 0.25 V and 0.3 V with 0.1 mL positive serums (●) spike, and other results of 0.2 mL anti-HBs containing (○) electrolyte solution. At these curves, 0.1 V amplitude responded sensitively to both peaks, where 8.47×10^{-8} A (serum) and 24.75×10^{-8} A (HBs) were obtained. In this condition, SW frequency variations were examined in ranges of 30 Hz, 50 Hz, 80 Hz, 100 Hz, 150 Hz, 200 Hz, 250 Hz and 300 Hz. As shown in Fig. 3(B), the serum is 80 Hz (12.28×10^{-8} A) and HBs is

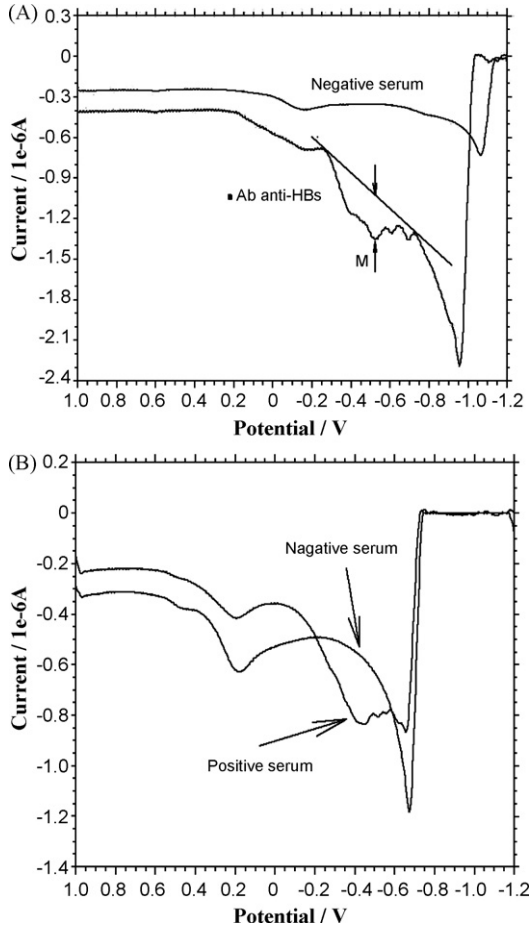

Fig. 2. BIDCE property, (A) comparison of healthy negative serum and anti-HBs spike. (B) Healthy negative and patient positive serum, using SW stripping voltammetry in 0.1 M H_3PO_4 electrolyte solution. Other parameters were used in optimized conditions.

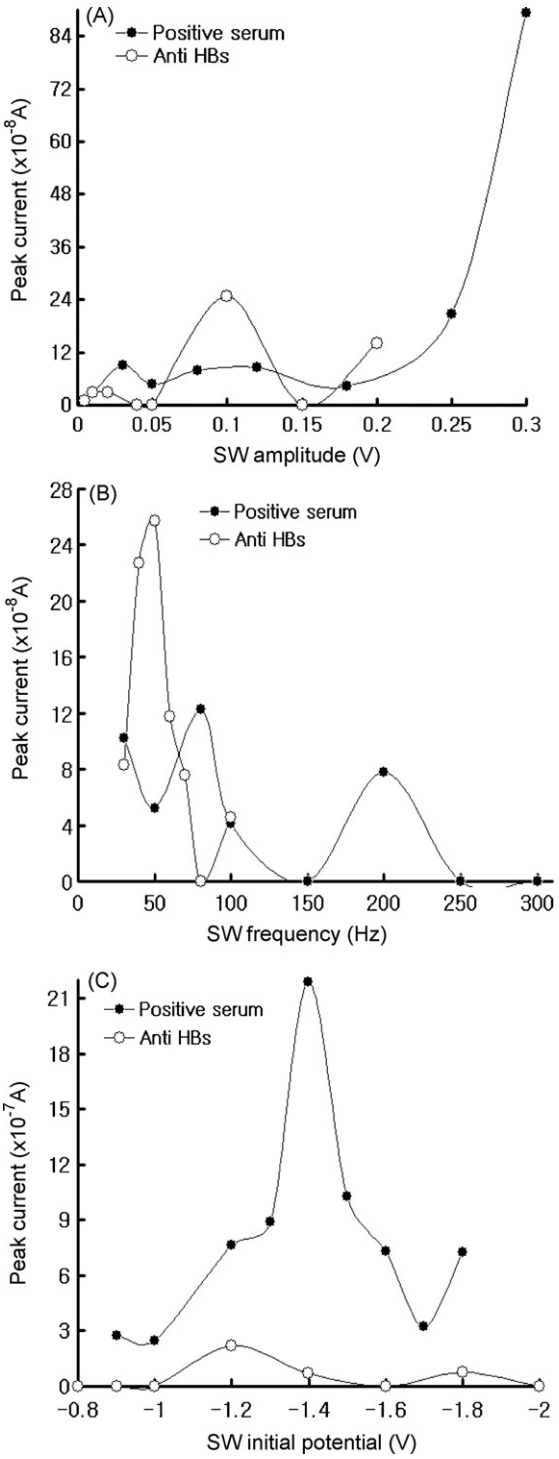

Fig. 3. (A) The variations of SW amplitude ranges from 0.01 V to 0.3 V. (B) Variations for SW frequency ranges from 30.0 Hz to 300.0 Hz. (C) Variations in SW accumulation potentials from −1.8 V to −0.9 V. Positive serums (●) containing 0.1 mL and 0.2 mL and anti-HBs (○) spike, 200 s accumulation time in 0.1 M H_3PO_4 electrolyte solution. Other parameters were used for the optimum conditions.

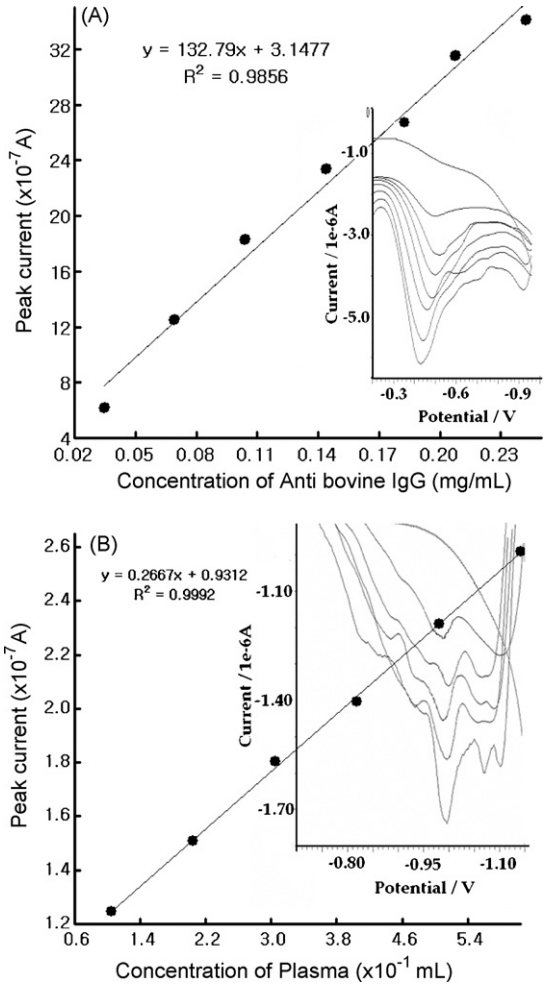

Fig. 4. (A) SW stripping working ranges of 0.035 mg/mL, 0.069 mg/mL, 0.104 mg/mL, 0.138 mg/mL, 0.182 mg/mL, 0.208 mg/mL and 0.242 mg/mL antibovine IgG in a 0.1 M $NH_4H_2PO_4$ solution with a pH of 7.7, SW amplitude of 0.1 V, SW frequency of 75 Hz, an increment potential of 0.04 V, an accumulation potential of −1.3 V, and an accumulation time of 100 s. (B) The concentration effects for 0.5 μL^{-1}, 1 μL^{-1}, 1.5 μL^{-1}, 2.0 μL^{-1}, 2.5 μL^{-1} and 3.0 μL^{-1} plasma in 0.1 M H_3PO_4, electrolyte solution and optimum conditions were set for other parameters.

50 Hz (25.75×10^{-8} A). Both peaks were sensitive at 75 Hz and were fixed. Here, SW accumulation potentials were then examined negative in ranges from −1.8 V to −0.9 V, with nine points. Fig. 3(C) shows the stripping peak current during −1.2 V to −1.4 V, HBs increased slowly for 2.89×10^{-7} A and positive serum increased sevenfold (22.5×10^{-7} A). HBs peak height and peak potential shifted to the negative direction, thus, −1.3 V accumulation potential was fixed. In this condition, SW accumulation time and the electrolyte pH strength were examined and obtained for 100 s accumulation time, pH 7.77. These results are not shown here. Thus, final parameters were fixed with 0.1 V amplitude, 75 Hz frequency, −1.3 V accumulation potential, 100 s accumulation time, and a 7.7 pH buffer solution. Here, SW working ranges were examined.

3.3. Analytical working ranges of serums and anti-HBs, statistics and interference

Under optimum conditions, analytical working ranges were examined using anti-HBs and patient human plasma. In Fig. 4(A), the working ranges were varied from 0.035 mg/mL to 0.242 mg/mL spiking at seven points using anti-HBs. The first curve is that of the electrolyte blank solution, in which no signal appeared. However, in the next addition, a small peak current appeared at 0.5 V, which continuously increased with a slope sensitivity of $\Delta x/\Delta y = 132.79$, at precision of $R^2 = 0.9856$. These curves can be used in diagnostic assay for HPV infection. In such conditions, human plasma sample was examined using patient serum. Fig. 4 (B) shows the sera test varying from 0.5 μL^{-1} to 3.0 μL^{-1} spike. In the electrolyte blank solution, 0.5 μL^{-1} patient's plasma was spiked, and 1.0 V identical peak potential was obtained. Thus, 1 μL^{-1}, 1.5 μL^{-1}, 2.0 μL^{-1}, 2.5 μL^{-1} and 3.0 μL^{-1} were spiked, the peak potential increased continuously, and the linear equation obtained was $y = 0.2667x + 0.9312$, at a precision level of 0.9992, and peak current varied for from 1.25×10^{-7} A to 2.55×10^{-7} A. Therefore, the method displayed sufficient sensitivity, indicating it can be useful in diagnostic applications. Statistics and interference effects were examined at optimum conditions, 0.1 mL positive plasma was spiked, then 15th repeated stripping was performed using 200 s accumulation time, which was obtained for 1.951×10^{-7} A to 1.651×10^{-7} A peak current with a precision of R.S.D. = 0.08. The peak signal was very stable, making analysis possible. Interfering ions were examined in 0.1 mL positive serum with 0.1 mL amino acid of tryptophan, histidine, isoleucine, lysine, tyrosine, leucine and glycine spike, whose % of interference was obtained for −5.87%, −72.07%, 26.71%, 294.97%, 96.05%, −40.29% and −36.56%, respectively. Lysine was very strongly affected. In this condition,

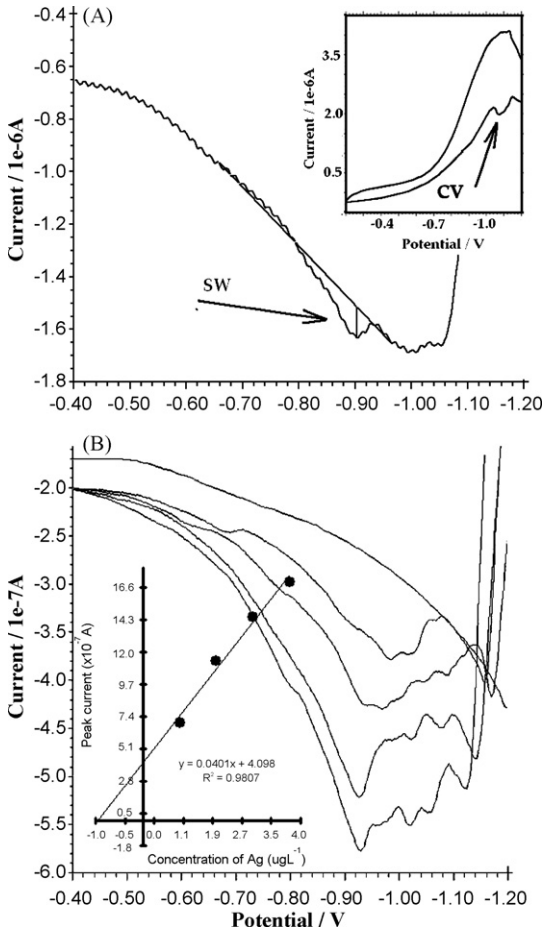

Fig. 5. Positive plasma test. (A) Mild case patient in SW stripping voltammetric conditions: 75 Hz frequency, 50 mV amplitude, −1.3 V accumulation potential, 100 s accumulation time, and 30 mV increment potential. CV conditions: 0.2 V initial, −1.2 V switching potential, 100.0 mV/s scan rate (insert curve). (B) The standard addition methods with patient sample, using optimum SW stripping conditions, each curve represents the electrolyte blank solution and the 0.05 mL positive plasma spike, then 0.2 mL, 0.4 mL, 0.6 mL anti-HBs spike.

diagnostic application was performed using patient plasma, with mild and severe case patients.

3.4. Patient diagnosis

Under optimum conditions, patient plasma was examined using SW and cyclic voltammetry, and serum sample was obtained from the National Blood Transfusion Research Institute. Fig. 5(A) shows SW for 0.05 mL serum spike in the electrolyte solution, accumulation time is 200 s. In the patient considered to be a mild case, −0.90 V peak potential was obtained with -1.183×10^{-7} A peak height in a positive scan. The other patients' sample was examined using CV scan with the same methods and producing the same result. Moreover, the mild and severe cases showed the same peak current. Fig. 5(B) shows the standard addition method using positive patients plasma, 0.05 mL raw plasma was spiked and a small peak was obtained. Standard anti-HBs were spiked continuously, then the linear curve was calculated, yielding 0.0036/0.05 mL.

4. Conclusion

A novel HBV bioassay was developed using a BIDCE sensor for bovine IgG-DNA-CNT linking bio electrode. The bovine IgG was immobilized with cyclic voltammetry, in which the BIDCE sensor was set at optimum parameters via SW stripping and CV scan. Linear analytical parameters were attained for 0.5–3.0 μL^{-1} HBV plasma and for 0.035–0.242 mg/mL anti-HBs in a 0.1 M $NH_4H_2PO_4$ electrolyte solution. The results can be applied to positive patient serums and HBV infection analysis. Moreover, diagnostic applications for non-treated blood serums are sensitively detected. This method can be used in real-time medical diagnosis as well as in direct *in vivo* monitoring.

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