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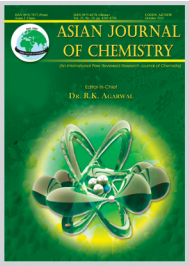

Direct Electrochemical Detection of Trace Porcine Insulin on Carbon Paste Electrode with Modified Graphite Powder

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Macromolecular organic species generally shows slow electron-transfer rate and produces insensitive redox peaks on solid electrodes. In this work, electrochemical behaviour and analytical detection of porcine insulin were performed using carbon paste electrode. It was found that insulin could adsorb on the carbon paste electrode surface effectively, which resulted in the enhancement of its direct electron transfer rate. Furthermore, insulin exhibited a sensitive anodic peak at about +0.5 V on the carbon paste electrode and its electrochemical process is irreversible completely. Under the optimized conditions (*i.e.* 5×10^{-3} M sodium carbonate supporting electrolyte with pH 10.50, 30 % paraffin oil and 70 % graphite powder for $\Phi = 2.5$ mm electrode, accumulation at the open-circuit potential for 60 s), the anodic peak current was good linear to insulin concentration in the range from 50 to 700 nM with a correlation coefficient of 0.997. The limit of detection was calculated to be 10 nM (S/N = 3). This result was successfully applied to the determination of insulin in medical injection and the recovery was 96.4-104.3 %.

Key Words: Electrochemical behaviour, Electroanalytical detection, Insulin, Carbon paste electrode.

INTRODUCTION

Direct electrochemical detection of insulin is of considerable interest because it is not only an essential hormone, which restricts the glucose levels in blood with a very narrow range and also important for the treatment of insulin-dependent type I diabetes[1]. It is well-known that some ways like bioassays[2], immunoassays[3,4] and chromatographic methods[5-7] are three standard analytical procedures for insulin detection. These sophisticated technologies have the disadvantages of high cost and low efficiency. HPLC with UV-VIS[8], fluorescence spectroscopy[9,10] and mass spectrometry with isotope dilution assay (IDA)[11] have also been developed for its detection. They are typically complicated analytical methodologies that frequently require derivatives of insulin with stable isotopes or fluorogenic labels to increase the sensitivity of detection and expensive instruments. In recent years, capillary electrophoresis separation technique[12-14] has become an attractive alternative for the insulin analysis since its high separation efficiency. Electrochemical methods have also attracted a great deal of attention due to its advantages of low cost, high sensitivity and easy-to-use and generally promote the analytical methodologies for this polypeptide.

The insulin molecule consists of two polypeptide chains linked by two disulphide bridges, A chain of 21 amino acid residues and B chain of 30 amino acid residues. Electrochemical detection of insulin mainly depends on either a reduction of some of these bonds at the mercury[15] and silver[16] electrodes or their oxidation at modified electrodes[17]. Currently, various chemically modified electrodes have been suggested to promote the oxidation and to detect it. Cox and Gray[17] reported the electrochemical detection of bovine insulin based on a ruthenium dioxide-cyano-ruthenate film accelerated the insulin oxidation in acidic environment. Then several groups inspired from the redox mediators and reported the insulin tested by RuOx complexes[18,19], polynuclear RuO-RuCN[20], chloro-complexes of iridium[21], iridium oxide[22] and ruthenium metallodendrimer[23]. Furthermore, other specific mediators such as CNTs[24], $K_4[Mo(CN)_8]$+Ni[25], RuOx/CNTs[26], Ni microparticles[27], SiC nano-particles[28] and CNT/$NiCoO_2$[29] have also been used for electrode modification in measurement of insulin.

These modified electrodes have been successfully employed for detecting the insulin. However, some matter like the leaching of the electron transfer mediator, poor long-term stability of mediators and electrocatalysts under alkaline medium condition, high detection limit and complicated multi-

step preparation methods by using specific as well expensive reagents have also appeared. Hence less expensive insulin complex detector with minor limitations is a developing way to the future[21].

Carbon paste electrodes (CPE) have some advantages, such as non-toxic, low background currents, wide potential windows and high active superficial area[30,31]. Recently, the carbon paste electrode has been widely utilized to detect a number of important electroactive biological molecules, including some polypeptides[32] and proteins[33,34]. In this work, in order to get high detection sensitivity and low detection limit with a wide linear range, here we used the differential pulse voltammetry (DPV), an analytical method has been conducted to measure porcine insulin. The result indicated that the developed carbon paste electrodes, based on the good adsorptive property of activated graphite powder surface, possessed obvious advantages including high sensitivity, excellent selectivity, good reproducibility and easy renewability in alkaline medium. Additionally, this new method would contribute to a faster and more accurate way of pharmaceutical analysis to detect porcine insulin *in vitro*.

EXPERIMENTAL

Porcine insulin (INS, ≥ 27 USP units/mg, m.w. = 5778) and its injection were purchased from Xuzhou Wanbang Biochem. Pharm. Co. Ltd., (Jiangshu, China). High purity graphite powder (30 μm ≤ ϕ ≤ 100 μm) and paraffin oil were purchased from Shanghai Colloid Chemical Engineering Co. Ltd., China. All other chemicals were analytical reagent grade from Beibei Chemical Reagent Co. (Chongqing, China) and used without further purification. The standard stock solution of 0.1 mM porcine insulin and 1.0 M Na_2CO_3 were prepared using deionized water. 5 mM Na_2CO_3 buffer solution has been adjusted to an appropriate pH value using 1.0 M NaOH and 1.0 M HCl.

Graphite powder pretreatment: The graphite powder was cleaned with acetone, rinsed with deionized water and then activated with aqua regia for 0.5 h. Then the graphite particles were washed with water until a neutral pH was reached and then dried in an oven at 200 ºC.

Working paste electrode preparation: The paste was prepared by thoroughly hand mixing 0.35 g of activated graphite powder (70 %, w/w) with 0.15 g of paraffin oil (30 %, w/w) in 10 mL weighing breaker and a portion of the composite mixture was packed into the end of a Teflon tube (about 2.5 mm *i.d.*, 3 mm depth). Electrical contact was made by forcing a same inside diameter copper down into the Teflon and into the back of the composite. The tip of the electrode was polished with a piece of weighting paper and then rinsed with redistilled water.

Cyclic voltammograms (CVs) and differential pulse adsorptive stripping voltammograms (DPASVs) were performed using a CHI660A electrochemical workstation (CH Instruments Inc., American). A three-electrode system was used with carbon-paste-working electrode, a saturated Ag/AgCl reference electrode and a circular Pt wire as the counter electrode. All the voltammetric experiments were preformed in the buffer solutions including different porcine insulin concentration at ambient temperature. A digital pHS-25C precision pH/mV meter from Kangyi Apparatus Co. Ltd. (Shanghai, China) was applied for the preparation of different pH buffer solutions in experiments.

Analytical procedure mainly contained two steps: Accumulation and determining. Firstly, porcine insulin was preconcentrated on the carbon paste electrode surface under an open-circuit potential for time (t_{acc}) = 60 s stirring in view of the results obtained in Section 3.3. Then, porcine insulin was oxidized during the following differential pulse voltammetry sweep from 0.1 to 0.8 V, resulting in a sensitive oxidation peak at 0.41 V, which measured as the analytical signal for porcine insulin. Furthermore, the optimized parameters of differential pulse voltammetry were as follows: pulse amplitude E_p = 50 mV, scan rate ν = 50 mV/s and pulse width t_p = 50 ms.

RESULTS AND DISCUSSION

Electrochemical behaviour of porcine insulin on the carbon paste electrode: Porcine insulin was characterized by the excellent electrochemical behaviour on the carbon paste electrode. In Fig. 1A, curve 'a' shows the cyclic voltammogram obtained from carbon paste electrode in the blank Na_2CO_3 buffer solution (pH 10.0), the redox peaks have not been observed. However, the voltammogram of 2.0×10^{-6} M porcine insulin on the carbon paste electrode under similar conditions exhibited only one anodic oxidation peak near 0.50 V with no peak on the reverse scan, indicating the totally irreversible nature of the electrode reaction of porcine insulin (curve 'b'). In addition, the anodic peak was susceptible to oxidation of tyrosine in porcine insulin according to the reported results[32]. To approach the reasons for adsorptive reaction on porcine insulin oxidation, certain important complementary experiments were performed. The carbon paste electrode was immersed into a 2.0×10^{-6} M porcine insulin solution for 60 s accumulation and rinsed with distilled water softly and its response was measured in a blank solution. Similar porcine insulin oxidation peak (curve 'c') with the peak current inferior to curve 'b' was observed, which is possibly due to adsorption effect of porcine insulin on the carbon paste electrode surface. Under the identical experimental condition with curve c, when bare glass carbon electrode (GCE) was used to check the oxidation of porcine insulin by cyclic voltammetry, the peak response signal hardly occurred (not shown).

In order to provide further more information about the absorptive characteristics of porcine insulin on the carbon paste electrode surface, the electrochemical behaviour of 2.0×10^{-6} M porcine insulin on the carbon paste electrode surface was compared by differential pulse voltammetry. The result shown in Fig. 1B was similar to cyclic voltammograms. No oxidation peak for the response of blank solution on the carbon paste electrode was observed (curve a_1) and an outstanding oxidation peak of 2.0×10^{-6} M porcine insulin was significantly ocurred at about 0.45 V (curve b_1). Under the identical conditions, the carbon paste electrode after 60 s accumulation of 2.0×10^{-6} M porcine insulin solution at an open-circuit potential gave homogeneously increased response superior to curve a1 at about 0.43 V in blank solution (curve c_1). The adsorption of porcine insulin at carbon paste electrode can be used as an efficient preconcentration step prior to the electrochemical oxidation of the surface species and the differential pulse voltammetry method was also chosen.

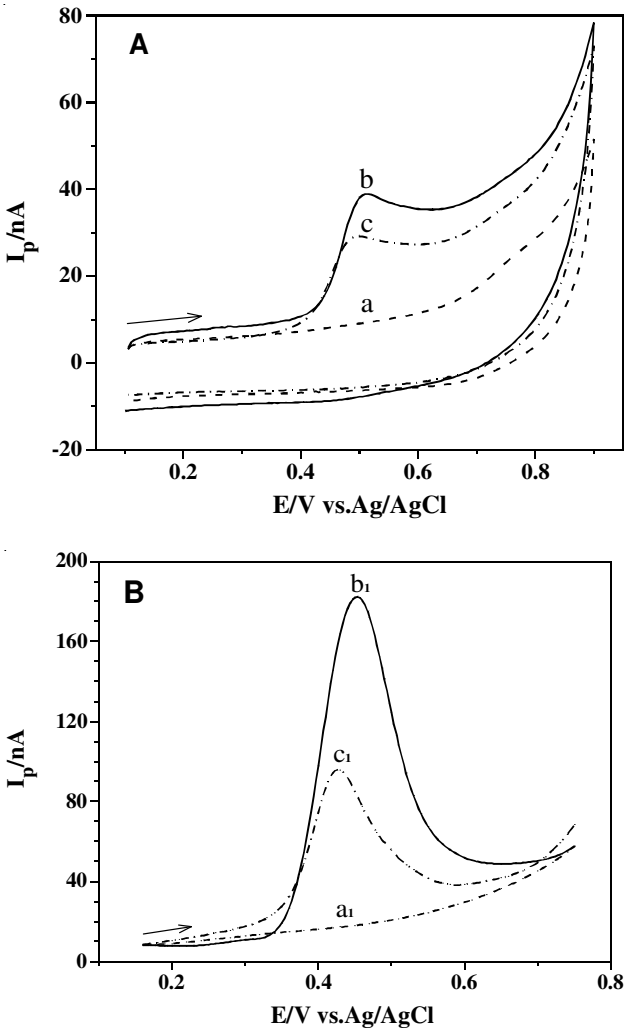

Fig. 1. (A) Cyclic voltammograms obtained from carbon paste electrode in the blank buffer solution (a) and 2.0×10^{-6} M porcine insulin solution (b) and carbon paste electrode in a blank solution after 60 s accumulation in a 2.0×10^{-6} M porcine insulin solution on the open-circuit potential (c). The cyclic voltammogram data were measured at scan rate of 50 mV/s. The buffer solution was 5.0×10^{-3} M Na_2CO_3 of pH 10.0. (B) as (A) for DPASVs of carbon paste electrode in the blank solution (a_1, c_1) and porcine insulin solution (b_1)

The electrochemical behaviour of 1.0×10^{-6} M porcine insulin solution (pH 10.0) at different scan rates of 30-110 mV/s was also investigated on the carbon paste electrode surface by cyclic voltammetry. The anodic peak current (I_{pa}, nA) varied linearly with increasing potential scan rates directly (Fig. 2), suggesting that the electrode process of porcine insulin follows a adsorption-controlled mechanism with linear regression equation:

$$I_{pa}/nA = 0.4387\nu/m\ V/s - 10.6929\ (R = 0.995)$$

Possibility to these properties of porcine insulin on the carbon paste electrode has been attributed to large specific adsorptive effect of modified graphite powder surface, just being similar to the previous reported some peptides[32] and proteins[35].

Determination of the electrochemical active surface area and surface adsorptive capacity: The electrochemical active surface area of the carbon paste electrode in a 2×10^{-3} M $K_3[Fe(CN)_6]$ and 0.1 M KCl solution was estimated by cyclic voltammetry. The Randles-Sevcik equation for the current-determining reaction of electroactive species $[Fe(CN)_6]^{3-}$ according to $[Fe(CN)_6]^{3-} + e = [Fe(CN)_6]^{4-}$ is[36]:

$$I_{pc} = (2.69 \times 10^5)\ n^{3/2} A D^{1/2} \nu^{1/2} C_0 \quad (1)$$

where, A, D, C_0 refer to the effective surface area (cm^2) of carbon paste electrodes, diffusion coefficient (cm^2/s) and concentration (mol/cm^3) of $[Fe(CN)_6]^{3-}$, respectively. Based on eqn. (1), A was calculated as 5.179×10^{-2} cm^2 at 298 K.

To infer more about the adsorbed capacity of porcine insulin on working electrode surface, chronocoulometry experiments were investigated in the presence of 2.0×10^{-6} M porcine insulin. The Cottrell equation for the total charge Q is shown as[37]:

$$Q = 2nFAcD^{1/2}t^{1/2}/\pi^{-1/2} + Q_{dll} + Q_{ads} \quad (2)$$

The adsorbed capacity (Γ) can be evaluated through the following equation:

$$Q_{ads} = nFA\Gamma \quad (3)$$

where, n, c, D refer to the number of electrons transferred, substrate concentration and diffusion coefficient of porcine insulin in solution, respectively. On the other hand, A is the electrochemical active surface area of carbon paste electrode, Q_{dll} is the double layer charge and Q_{ads} is the adsorption charge. Other symbols are used as their typical value. The Q_{ads} could be obtained from the intercept of the Anson's plots (Q *versus* $t^{1/2}$), assuming that Q_{dll} remained unchanged. Based on eqn. (3), when n is 2, the surface adsorbed capacity (Γ) of porcine insulin on the carbon paste electrode was calculated as 1.02×10^{-11} mol/cm^2.

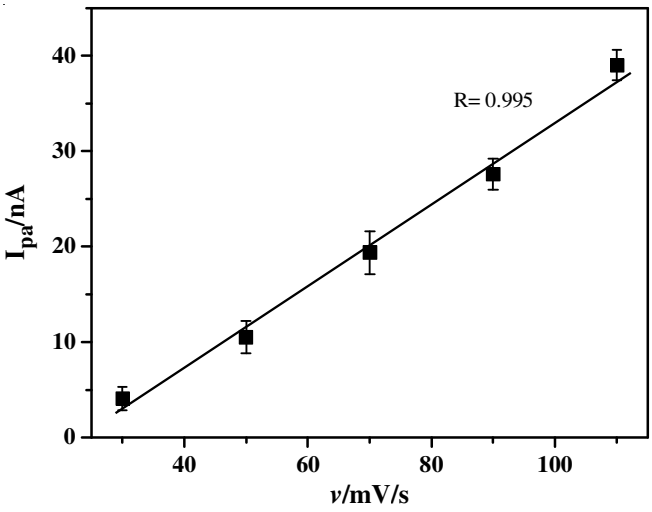

Fig. 2. Plot of oxidation peak current of 1.0×10^{-6} M porcine insulin *vs.* potential scan rates lower than 110 mV/s. The error bars represent the standard deviation of repetitive measurements (n = 3). Other conditions are the same as in Fig. 1

Optimum parameters to monitor porcine insulin by differential pulse voltammetry: For selecting the best mode of graphite powder modification, H_2O_2 (30 % w/w), high temperature (400 ºC) and aqua regia were used to activate the graphite for 30 min, respectively. The anodic DPV peak response of 1.0×10^{-6} M porcine insulin solution (pH 10) at different carbon paste electrodes prepared with graphite powder after modification of different modes and paraffin oil was compared. Furthermore, the influence of the mass ratio of graphite powder and paraffin oil in the carbon paste on the

response signal was also investigated by differential pulse voltammetry. It was found that the maximum differential pulse voltammetry peak response was obtained on the carbon paste electrode with the mixture of graphite powder modified by aqua regia (70 %, w/w) and paraffin oil (30 %, w/w).

The influence of supporting electrolyte on the response of porcine insulin on the carbon paste electrode was also explored (not shown). After testing it in different solutions including Na_2SO_4, Na_2CO_3, K_2SO_4 and K_2CO_3, the Na_2CO_3 solution was chosen for porcine insulin detection because of its highest DPV peak current under the same conditions. In addition, the effect of ionic strength on the peak current has been studied by changing the concentration of Na_2CO_3 solution from 1×10^{-4} M to 5×10^{-1} M (Fig. 3). It was observed that porcine insulin exhibited the largest DPV peak current at carbon paste electrode in the 5.0×10^{-3} M of Na_2CO_3 solution.

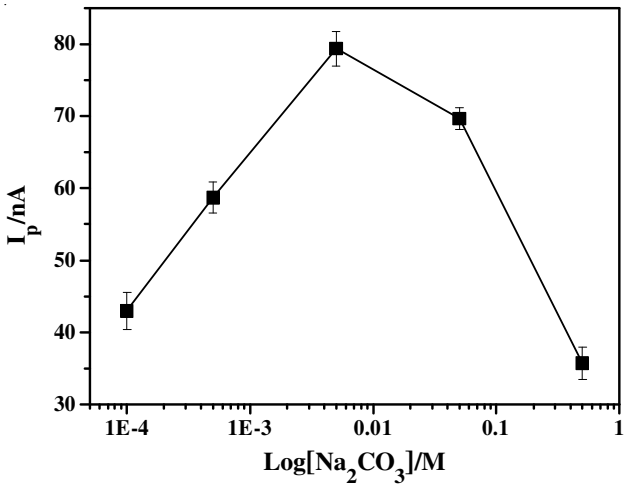

Fig. 3. Plot of DPV peak current of 9.6×10^{-7} M porcine insulin *vs*. the logarithm of various concentration of Na_2CO_3 solution from 1×10^{-4} to 5×10^{-1} M at the same pH 10.80. The error bars are similar to Fig. 2

The choice for accumulation condition of porcine insulin on the carbon paste electrode surface had significant effect on the determining sensitivity and detection limit. This work revealed that the DPV peak current was near the maximal value under the open-circuit potential and almost independent of accumulation potential in the range of -0.1 to 0.9 V for pH 10.50, indicating that the accumulation is mainly derived from the spontaneous adsorption of porcine insulin onto graphite powder surface and almost independent of electrostatic attraction, which is in agreement with the results described above. Meanwhile, the dependence of DPV peak current of porcine insulin on the accumulation time, t_{acc}, over the range of 20 to 80 s was also checked at the working carbon paste electrode in 5.0×10^{-3} M Na_2CO_3 at pH 10.50 (Fig. 4A). The peak current grew with prolonging accumulation time. For 4.5×10^{-7} M porcine insulin solution, it still grew when the accumulation time was up to 80 s, although the increase became quite slow after 60 s, suggesting that a saturated adsorption is nearly achieved. In the following experiments, the t_{acc} was fixed at 60 s on open-circuit condition.

The influence of pH of the supporting electrolyte on the peak current and peak potential of 5.0×10^{-7} M porcine insulin is presented in Fig. 4B. When the solution pH changed from 8 to 12, the DPV peak current increased at first and then decreased rapidly and it achieved a maximum value around pH 10.50. Furthermore, the porcine insulin oxidation peak potential shifted to positive value with pH decreasing. The average moving rate (*i.e.* slope) was about 67 mV/pH being close to the theoretical value of 59 mV/pH[36], indicating the participation of the same protons and electrons in the electrochemical process. Hence, the peak current also suffered from the effect of proton concentration to some extent. However, the concrete electrochemical reaction mechanism for porcine insulin oxidation on the carbon paste electrode in alkaline medium needed further research.

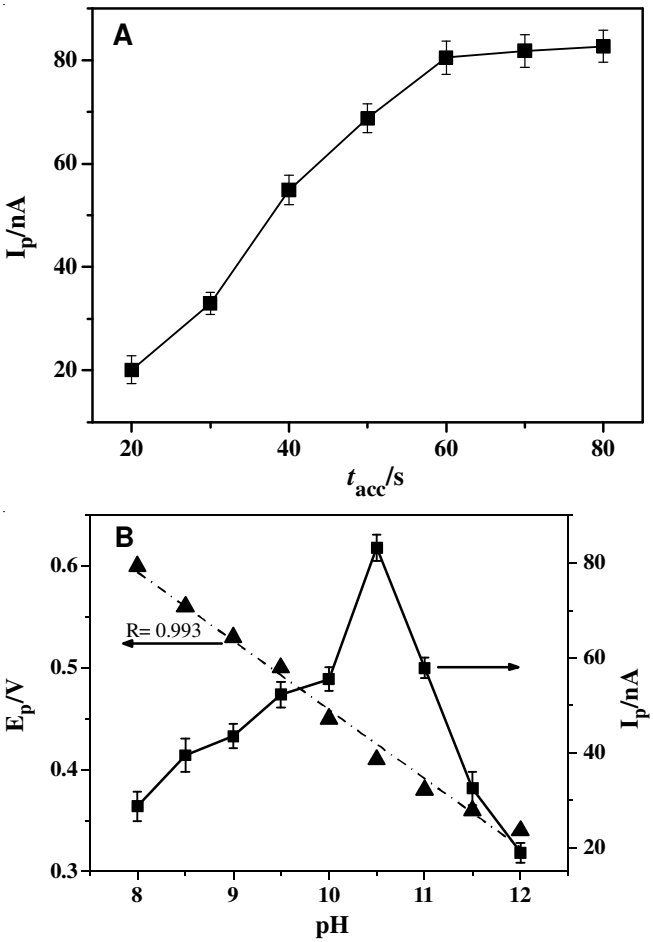

Fig. 4. (A) Effect of accumulation time on the DPV peak current of 0.45 μM porcine insulin solution (pH 10.50) at the open-circuit potential. (B) Effect of pH (8.0-12.0) on the oxidation peak potential and current of 5.0×10^{-7} M porcine insulin in DPV response. The error bars are similar to Fig. 2

Electroanalytical characteristics of carbon paste electrodes: Fig. 5 shows that the DPV peak current increased linearly with increment of porcine insulin concentration in the range from 5×10^{-8} M to 7×10^{-7} M. The linear regression equation was expressed as I_p/nA = 1.73535 nA +175.43 $C_{porcine\ insulin}$/μM (R = 0.997). The detection limit was 1×10^{-8} M (S/N = 3). According to previously reported results, detection limits of 2.3×10^{-8} M, 5×10^{-7} M, 2×10^{-8} M, 1.4×10^{-8} M and 3.8×10^{-8} M were obtained for porcine insulin at RuOx/CFME[18], RuO/RuCN/carbon fiber microelectrode (CFME)[20], IrOx/

GCE[22], CNT/GCE[24] and CNT/nickel-cobalt oxide/GCE[29], respectively. Hence, it can be concluded that the carbon paste electrode shows excellent sensitivity and lower detection limit for porcine insulin.

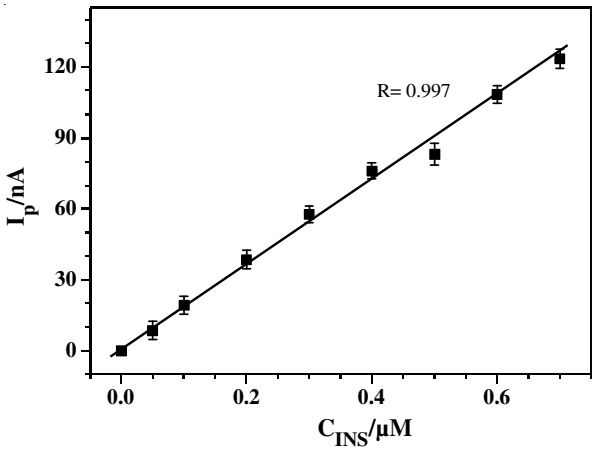

Fig. 5. Plot of DPV peak current response *vs*. porcine insulin (INS) concentration from 0 to 7.0×10^{-7} M at the open-circuit potential for t_{acc} = 60 s. The buffer solution was 5.0×10^{-3} M Na_2CO_3 of pH 10.5. The error bars are similar to Fig. 2

In addition, after each measurement, the used paste was carefully removed from the end cavity of the electrode and another new carbon paste electrode was fabricated again. 2×10^{-7} M of porcine insulin solution was measured for nine times with a carbon paste electrode renewed after every measurement and the relative standard deviation of peak current was 4.8 %. The electrode could be used for long-time owing to the stability of pretreated graphite matrix. Besides, some common co-existing organic complexes and metal ions in porcine insulin pharmaceuticals were tested to check their levels of interference in 2×10^{-7} M porcine insulin determination. The results suggested that at least 100-fold concentration of ascorbic acid, phenic acid and glycerine; 200-fold concentration of Ca^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+} had no influence on the signal of porcine insulin with deviations below 5 %.

Sample analytical application: To confirm the sensitivity and generality of the proposed differential pulse voltammetry method, here we have used this method for the determination of porcine insulin in its medical injection by using standard addition method. The porcine porcine insulin injection without further pretreatment was diluted appropriate times with 5.0×10^{-3} M Na_2CO_3 solution (pH 10.50). The results are listed in Table-1. The recovery was in the range of 96.4-104.3 %, indicating that this method has good accuracy.

TABLE-1
DETERMINATION RESULTS OF PORCINE INS IN ITS MEDICAL INJECTION AFTER APPROPRIATE DILUTION (n = 5)

Insulin added (nM)	Insulin found (nM, RSD < 3.8 %)	Recovery (%)
0	208.5[a]	104.3
50	241.0	96.4
100	303.0	100.9
150	349.2	99.8
200	391.8	97.9
250	454.6	101.0

[a]Expected concentration of insulin was 200 nM

Conclusion

In summary, here we report an excellent electrochemical sensor *via* simple and inexpensive carbon paste electrode, without any exceptional modifiers, for the DPASV nanomolar determination of porcine porcine insulin for the first time. The electrochemical and adsorptive behaviour of porcine insulin on the carbon paste electrode surface have been explained using cyclic voltammetry and differential pulse voltammetry. The electrochemical active surface area of carbon paste electrode was *ca*. 5.179×10^{-2} cm^2. The electrode exhibited attractive properties such as simplicity of electrode preparation, high reproducibility and sensitivity, a fast response and a distinct advantage of renewability. These advantages encouraged us to employ it as an electrochemical sensor in batch and flow systems. Therefore, the experimental results are promising for its applications in the chromatographic methods for routine pharmaceutical analysis.

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