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An Electrochemical Senor for Determination of Amlodipine Besylate Based on Graphene–Chitosan nanoComposite Film Modified Glassy Carbon Electrode and Application in biological and pharmaceutical samples

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The graphene–chitosan composite film modified glassy carbon electrode was fabricated and used to determine amlodipine besylate. In 0.1 M pH 7.3 phosphate buffer solutions, the redox peak currents of amlodipine besylate increased significantly at graphene–chitosan composite film modified glassy carbon electrode compared with bare electrode and chitosan modified glassy carbon electrode, indicating that graphene possessed electrocatalytic activity towards amlodipine. The experimental conditions were optimized and the oxidation mechanism was discussed. Under the optimal experimental conditions, the oxidation peak current was proportional to amlodipine concentration in the range from to 1-70 μM with the correlation coefficient of 0.9930. The detection limit was 0.6 μM (S/N=3). Using the proposed method, amlodipine was successfully determined in serum sample, tablets and urine, suggesting that this method can be applied to determine amlodipine in pharmaceuticals.

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Introduction

Calcium ions are required to generate electrical activity for the contraction of cardiac and smooth muscle and conduction of nerve cell. Calcium antagonist is a drug that inhibits the entry of excess calcium into cells and prevents the mobilization of calcium from intracellular stores, resulting in relaxation of blood vessel walls and cardiac muscle for blood to flow more freely. This causes lowering of blood pressure thereby reducing oxygen demand in the heart and relieving angina pain. Amlodipine besylate (ADB), 3-ethyl 5-methyl (4RS)-2-(2-aminoethoxy) methyl)-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzene sulphonate (Fig. 1) is arelatively new potent long-acting calcium channel blocking agent [1-3].

Amlodipine is a third-generation dihydropyridine calcium antagonist which is used alone or in combination with other medications for treating high blood pressure, certain types of vasospastic angina, hypertension, cardiac arrhythmias, and coronary heart failure [4-6].

ADB is available in the market as bulk material, tablets, capsules and compounded capsules; hence, it is important to have a rapid and simple analytical technique for the determination of ADB in pharmaceutical preparations and human body fluids.

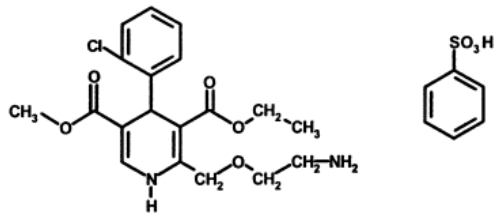

Fig. 1. The chemical structure of amlodipine besylate

Several analytical methods have been reported for the analysis of amlodipine besylate in human serum, including high-performance liquid chromatogramphy (HPLC) [7, 8] liquid chromatography (LC) [9], high-performance thin layer chromatography [10], gas chromatography (GC) [11], capillary electrophoresis [12], flow injection analysis [13], enzyme-linked immunosorbent assay [14], spectrofluorometric[15] and spectrophotometric methods [16, 17]. Recently, in order to obtain an effective separation and sensitive detection some coupled methods such as liquid chromatography coupled with tandem mass spectrometry [18, 19]and solid-phase extraction with cation-exchange column [20] have also been used to monitor the ADB concentration. Several of the spectrophotometric procedures published show disadvantages such as a heating or extraction step, narrow range of linear response and also need of special reagents. Chromatographic methods offer the required sensitivity and selectivity but need sample clean-up, complicated extraction, tedious and time-consuming derivatization procedures, relatively heavy and expensive instrumentation which limited their use in quality control laboratories for analysis of amlodipine in its pharmaceutical dosage forms and human body fluids.

As amlodipine besylate is an electroactive compound, which can be easily subjected to oxidation on different working electrodes, it can be investigated by electrochemical methods [21]. Compared with other methods, electrochemical methods have the advantages such as being simple, sensitive, and selective while with small amounts of sample. Also electrochemical sensors can be fabricated with small dimensions suitable for placement directly into biological samples. Yet, it is still of prime importance to search for new electrodes that do not need any treatment or pretreatment for prevention of electrode fouling.

In recent years, graphene nanosheets have attracted more and more attentions in the preparation of sensors and biosensors due to their large surface area, extraordinary electronic transport property, high electrocatalytic activity, good mechanical strength, high thermal conductivity and high mobility of charge carriers. However, the aggregation of graphene nanosheets limits their application [22]. In order to conquer this disadvantage, graphene nanosheets and chitosan are frequently used together because the positively charged chitosan can interact with the negatively charged graphene nanosheet to prevent their aggregation [23]. For instance, Li' group [24] reported the electrochemical determination of dopamine in the presence of ascorbic acid based on graphene nanosheets and chitosan modified GCE. Niu et al. proved that low-potential NADH detection can be achieved at an ionic liquid-functionalized graphene nanosheets and chitosan modified GCE [25]. It has also been reported that graphene nanosheets and chitosan can also be applied to investigate the direct electron transfer of glucose oxidase [26, 27], cytochrome c [28] and horseradish peroxidase [29].

In this paper, a simple and fast method for the determination of amlodipine besylate (ADB) at graphene–chitosan composite film modified glassy carbon electrode (GR-CS/GCE) was presented by

using cyclic differential pulse voltammetry. The electrochemical behavior of ADB was investigated with cyclic voltammetry. The performance of the fabricated electrode, such as linear range and detection of limit, was evaluated and discussed.

Material and Methods

Apparatus

Electrochemical experiments were performed on a computer controlled μ-Autolab electrochemical system (Eco-Chemie. Utrecht, Netherlands) equipped with the GPES software. Cyclic voltammograms of ADB in phosphate buffer solution (PBS) of 7.4 were recorded at a scan rate of 50 mV s^{-1} from 0.2 to 1.5 V. All solutions were deaerated by bubbling nitrogen prior to the experiments and the electrochemical cell was kept under nitrogen atmosphere throughout the experiments. The electrochemical cell was assembled with a conventional three-electrode system consisting of saturated calomel electrode (SCE) as a reference electrode and platinum disk and as the counter electrode and a GR-CS/GCE as a working electrode. The surface morphology of modified electrodes was characterized with a scanning electron microscope (SEM, Philips XL 30).

Reagent and chemicals

Amlodipine besylate was from Amin pharmaceutical company (Esfahan, Iran). Graphene purchased from Sigma-Aldrich. All reagents were of the maximum available purity and were used without further purification. The stock solution of ADB (1 mM) was prepared in methanol/water (1:10). The studies were carried out in the pH range 2 to 10 using 0.1 M phosphate buffer solution prepared by mixing the stock solutions of Na_2HPO_4 and NaH_2PO_4, according to the method of Christian and Purdy [30]. Distilled deionized water was used throughout and working solutions were prepared freshly before the analysis. Nitrogen (99.99%) was used to remove dissolved oxygen.

Procedure

Preparation and Characteristics of the modified electrode

1mg graphene was added into1mL 0.5% chitosan solution and then sonicated for 4 h to give a homogeneous solution. The glassy carbon electrode (GCE) was polished with 0.3 and 0.05 μM alumina powders and rinsed thoroughly with doubly distilled water between each polishing step. After that, the GCE was sonicated in doubly distilled water, anhydrous ethanol and redistilled deionized water, and dried under N_2 blowing. Then, 5 μL of graphene–chitosan dispersion was dropped on GCE surface. After the solvent evaporated, the electrode surface was thoroughly rinsed with doubly distilled water to wash away the unimmobilized modifier and dried. The obtained electrode was noted as GR–CS/GCE. For comparison, the Chitosan/GCE and Graphene/GCE were fabricated with the similar procedure. The surface of GR–CS/GCE was characterized using scanning electron microscopy (SEM). Fig. 2 showed graphene-chitosan nano composite film looks like a thin wrinkling paper on electrode surface.

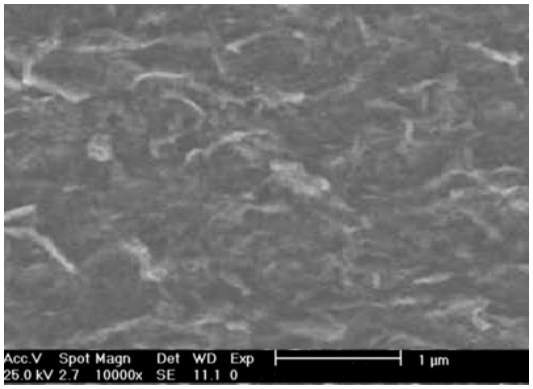

Fig. 2. SEM image of GR–CS/GCE

Electrochemical impedance spectroscopy (EIS) employed to determine the interface properties of the modified electrode. As shown, the Nyquist plot of the EIS measurement of GR-CS/GCE and bare GCE in the presence of equivalent 3.0 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ and 0.1 mol L^{-1}KCl. The frequency range is from 0.1 Hz to 100 kHz. It can be seen that only a very small semicircle with an almost straight tail line can be observed for a bare GCE (Fig. 3a) and a semicircular Nyquist impedance spectrum is observed for GR-CS/GCE (Fig. 3b). This phenomenon indicates that the electron transfer resistance at the electrode/electrolyte interface decrease after surface modification with graphene-chitosan composite film. Therefore, this also confirms the fact that graphene-chitosan composite

film had been attached to the electrode surface. Randles model was used as an equivalent circuit to fit all the data of electrochemical impedance measurement (insert in Fig. 3). Here, R_s, R, Q and Z_w represented Ohmic resistance of the electrolyte solution, the charge transfer resistance of the redox, constant phase element and Warburg impedance, respectively.

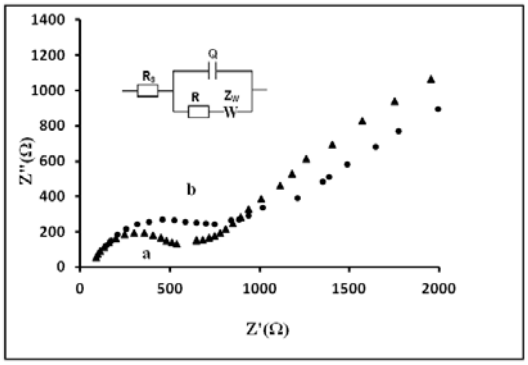

Fig. 3. Electrochemical impedance spectroscopy for the (a) bare GCE, (b) GR-CS/GCE in 3 mmol L^{-1}solution of $[Fe(CN)_6]^{3-/4-}$ in the presence of 0.1 M KCl.

Tablet preparation

Twenty tablets were weighed accurately and finely powdered. A portion of the powder, equivalent to 10 mg of amlodipine besylate was transferred to a 100 ml volumetric flask and dissolved in approximately 80 ml of methanol/water (1:10). The mixture was sonicated for 30 min and then completed to the mark with the same solvent. The solution was then filtered and the first portion of the filtrate was discarded. An accurate measured volume of the filtrate was quantitatively diluted with doubly distilled water to yield a sample solution having a final concentration assumed to be 1×10^{-4} M amlodipine besylate. An aliquot was then transferred to a voltammetric cell containing 10 ml of PBS (pH 7.4) to yield a final concentration of 1×10^{-6} M amlodipine besylate. The differential pulse voltammogram was then recorded. The nominal content of tablets is determined either from the calibration graph or from corresponding regression equation.

Urine and serum preparation

Urine samples, 1 ml each, were spiked with vary amounts ranged from 20 to 100 µg of amlodipine besylate. The analysis was carried out by adding 0.1 ml of amlodipine besylate urine solution to the electrochemical cell containing 9.9 ml of PBS (pH 7.4). The differential pulse voltammograms were recorded according to procedure described in Section 2.3.2. The procedure was completed as described for spiked urine samples.

Serum samples were spiked with convenient amounts of amlodipine besylate stock solution. A 1 ml portions of spiked serum were analyzed according to procedure described above.

Results and Discussion

Electrochemical behavior of amlodipine besylate

Cyclic voltammetry

Cyclic voltammograms were recorded for 50 µM ADB in 0.1M PBS solutions (pH 7.4) using bare glassy carbon electrode, GR-CS/GCE at scan rate 50 mV/s. The bare GCE showed any response towards the amlodipine besylate oxidation. After modification with graphene-chitosan composite film a well-defined peak appeared at anodic peak potentials of 580 mV for ADB oxidation with remarkable enhancement in peak current values as compared to bare glassy carbon electrode. Absence of reduction peak on reverse sweep clearly indicates that amlodipine besylate is irreversibly oxidized at GR-CS/GCE. The marked enhancement in the anodic peak current of amlodipine oxidation confirm the electrocatalytic effects of the graphene-chitosan towards the oxidation of ADB .Fig. 4 compares cyclic voltammograms of supporting electrolyte in the absence of ADB using GR-CS/GCE (curve a) and in the present of 100 µM ADB using GR-CS/GCE (curve d) at scan rate 50 mV/s. Fig. 3 clearly indicates that GR-CS/GCE serves as a better sensor in comparison to bare electrode in terms of higher current.

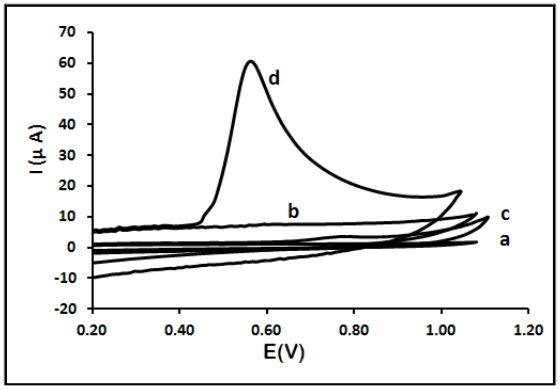

Fig. 4. Cyclic voltammograms of supporting electrolyte in the absence of ADB (a) and (b) and 50 μM ADB at the bare GCE (c) and GR–CS/GCE (d) in PBS of pH 7.4 at a scan rate of 50 mV/s.

The analytical characteristics of the proposed method were compared with the other similar electrochemical methods. Rajendra N. Goyal and Sunita Bishnoi determined amlodipine besylate in human urine and pharmaceuticals and cyclic voltammograms were recorded using bare, MWNT and SWNT modified EPPGE at scan rate 50mV/s. With the bare EPPGE, a broad oxidation peak (small bump) at peak potential ~700 mV with very low current value indicates that the bare EPPGE shows very poor response towards the amlodipine oxidation. After modification with multi-walled and single-walled nanotubes a well-defined peak appeared at anodic peak potentials of~605 mV and ~524mV respectively for amlodipine oxidation with remarkable enhancement in peak current values as compared to bare pyrolytic graphite electrode. In our study window potential and anodic peak potentials was lower than others.

Effect of scan rate

The effect of scan rate on the oxidation of 50 μM of ADB was examined in the range 10 to 300 mV/s at pH 7.4 using GR-CS/GCE by cyclic voltammetry as shown in Fig. 5.

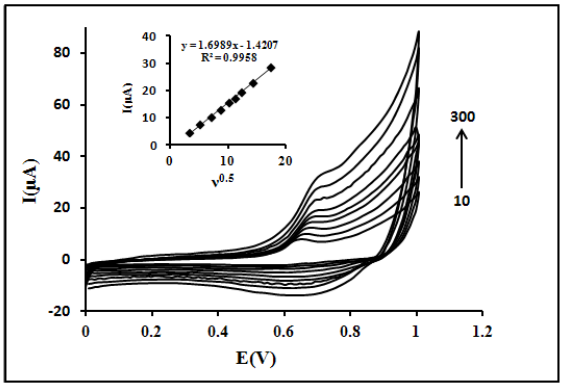

Fig. 5. Effect of scan rate from 10 to 300 mV s^{-1} on electrooxidation of 50 μM ADB in a 0.1 M PBS of pH 7.4 at GR-CS/GCE

The peak current of 50 μM ADB solution at the GR-CS/GCE increased linearly with the increase in scan rate and this behavior indicates the direct electron transfer on the surface of the modified electrode and ADB .Fig.5 (inset) presents a linear plot between peak current and scan rate having correlation coefficients of 0.9725. The linear relation between Ip and $v^{0.5}$ can be represented by Eq (1):

$I_p/\mu A = 1.698\ (v^{0.5}/mV\ s^{-1}) - 1.420$ (1)

These observations suggest that the diffusion plays a significant role in the irreversible electrode reaction of ADB at the GR-CS/GCE.

Effect of pH

The electrochemical behavior of ADB was investigated using phosphate buffer in the pH range 2 to 10. The influence of pH on the oxidation peak of ADB was examined using GR-CS/GCE by CV. It is observed that with increase in pH the peak potential shifts towards less positive values, which indicates the participation of protons in the electrode process. The slope of the linear E_p, pH equation was ~58 mV and suggested that equal number of protons and electrons is involved in the electrode reaction at the modified electrodes.

Detection limit

The variation of oxidation peak current with ADB concentration was studied using GR-CS/GCE. Various concentrations of ADB were prepared within range 0.01 to 0.5 mmol L^{-1} and cyclic voltammograms were recorded (Fig. 6).

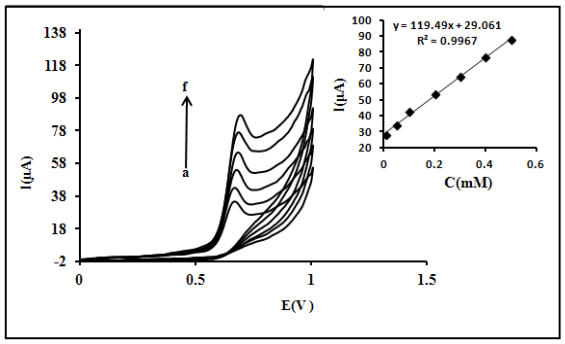

Fig. 6. Cyclic voltammograms of ADB in a pH 7.4 PBS buffer solution containing 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM ADB (from inner to outer) with the scan rate as 50 /mVs^{-1}

Fig. 7 illustrates a series of differential pulse voltammograms obtained for ADB were prepared within range 1 to 70 µM using GR-CS/GCE in 0.1 M PBS solution at pH 7.4. When the concentration of ADB gradually increases, the oxidation peak current (I_p) increases at the modified electrode as shown in Fig. 7.

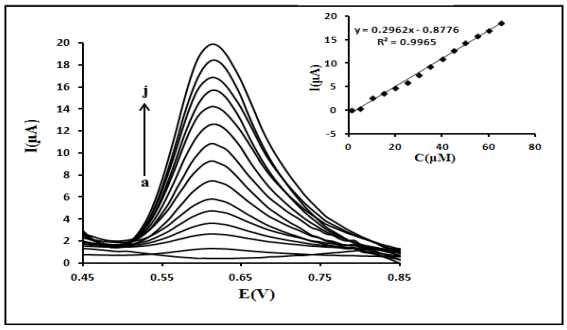

Fig. 7. Differential pulse voltammograms of ADB. Concentration (1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70µM) (from inner to outer); inset: the $i_p$versus*c* plot

The dependence between the peak current (Ip) and concentration can be expressed by Eq. (2):

$$I_p/\mu A = 0.2962\ ([\text{Amlodipine}]/\mu M) - 0.8776 \qquad (2)$$

This equation has correlation coefficients 0.9965. The detection limit was calculated by using the relation $3S_b/m$, where S_b is the standard deviation of the blank and m is the slope of the calibration curve and found to be 0.6µM.

Real sample analysis

The proposed method was applied to the determination of amlodipine besylate in commercial Norvasc® tablets. The percentage recoveries based on five separate determinations are presented in Table 1.

Table 1. Application of the proposed method to the determination of ADB in Norvasc® tablets

Sample	Claimed amount	Detected amount	Recovery%
Norvsac® tablets (5 mg per tablet)	5.0 mg	4.6 mg	92
Norvsac® tablets (10 mg per tablet)	10 mg	9.3 mg	93

The R.S.D. value for determination was less than 3.8% for n=5

The high sensitivity of the method allows the determination of amlodipine besylate in spiked human urine. The results are shown in Table 2. The results are satisfactorily accurate and precise.

Table 2. Application of the proposed method to the determination of ADB in spiked human urine

Add (µg/mL)	Found (µg/mL)	Recovery%
30	28.2	94
50	47.6	95.2
60	56.1	93.5

Mean ± S.D = 94.2 ± 0.873

The amount of ADB in serum was calculated from standard addition method. The precision and accuracy of ADB assay in serum were assessed from five replicates at five nominal concentrations (Table 3). The precision is expressed as the R.S.D. (%) and accuracy as a mean relative error. These values were acceptable. Good recoveries of ADB were achieved from serum.

Table 3. Precision and accuracy for assay of amlodipine besylate in serum sample

Added (µg/mL)	Found (µg/mL) mean ±S.D.	Recovery (%)	R.S.D. (%)	Mean relative error (%)[a]
20	18.6 ± 0.29	93	0.73	-7
40	38.2 ± 0.127	95	0.36	-4.5
50	47.3 ± 0.231	94.6	0.48	-5.4
60	58.4 ± 0.372	97.3	0.65	-2.6

a: mean relative error(%) = (measured concentration − nominal concentration) /nominal concentration×100

Interferences study

As the major metabolites present in human urine are uric acid and ascorbic acid which can interfere in the detection of analyte because of being redox active therefore, the possible interferences of these compounds on the oxidation peak of amlodipine have been evaluated to examine the selectivity of the proposed method. The experimental results show that up to 1000-fold excess of uric acid and ascorbic acid, they have no interference on the differential pulse voltammetric determination of 1×10^{-5} M amlodipine using GR-CS/GCE. The interference of major metabolite of amlodipine, 2-([4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycar-bonyl-6-methyl-2-pyridyl] methoxy) acetic acid has also been examined and it was found that it does not interfere in the determination of amlodipine up to 100-fold excess. The metabolite is not oxidizable hence, does not give any oxidation peak. The analysis of the obtained results indicates that these compounds do not interfere in the determination of amlodipine using the proposed method.

Reproducibility and stability

The reproducibility of the proposed method was evaluated by assaying GR-CS/GCE by measuring the oxidation current response for fresh solution of 1.0×10^{-6} M amlodipine for eight replicate measurements. The peak current was almost identical after eight continuous potential scans and compared to the original oxidation current value a discrepancy of only ±1.6% was observed. This suggests that the proposed method possesses good reproducibility.

The stability of the GR was also investigated. A voltammetric response of a fixed concentration of ADB at pH 7.4 was recorded daily over a period of 15 days. The observed results indicated that the voltammetric response after 15 days shows relative standard deviation (RSD) of 2.78%, which demonstrates the adequate stability of the GR-CS/GCE and indicates it to be very good sensor for the voltammetric detection of ADB.

Conclusion

In this study, an electrochemical sensor based on Graphene–Chitosan Composite Film Modified Glassy Carbon Electrode was used to investigate the electro-oxidation behavior of ADB in 0.1 M PBS of pH 7.4. Compared with unmodified GCE, the peak current for electro-oxidation of the drug was increased significantly at the modified electrode. Under the optimized experimental conditions, the calibration range and limit of detection of the proposed sensor were 1-70 µM and 0.6 µM, respectively**.** The sensor revealed considerable selectivity for ADB over several common drugs (e.g., acetaminophen, propanalol and atenolol) that usually consumed in accompany

with ADB, as well as the common interferences present in biological samples (e.g., ascorbic acid, glucose, tyrosine and cysteine) for the determination of ADB in biological fluids. The proposed voltammetric sensor possessing high sensitivity, short response time, low cost and high stability for months without any significant divergence was successfully applied to the determination of tablets and, more importantly, to monitor the therapeutic or toxic levels of the drug and to investigate its pharmacokinetics in serum samples and urine samples.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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