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Development of Electrochemical Platform Based on Carbon Nanotubes Decorated with Zirconium Oxide Nanoparticles for Determination of Nebivolol

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Electrochemical sensor based on carbon nanotubes decorated with zirconium oxide nanoparticles was developed for the determination of nebivolol at the potential of +1.05 V (*vs.* Ag/AgCl). Nanomaterials were dispersed in an anionic polymer and deposited by simple one-step casting method on glassy carbon electrode. Scanning electron microscopy and energy dispersive X-ray spectroscopy were used to study the surface morphology and structure characterization of deposited film. The sensor has a strongly improved sensitivity (9.29×10^{-6} μA M^{-1}). The electrocatalytical peak current of nebivolol shows a linear response from 100 nM to 10 mM with detection limit of 12 nM. Low-cost analysis of drug in serum samples was carried out after adsorption of nebivolol at the nanocomposite using differential pulse voltammetry. In comparison with HPLC method, the electrochemical method ensures faster and simpler quantification of nebivolol in pharmaceutical samples.

Keywords: Nebivolol, Zirconium oxide nanoparticles, Carbon nanotubes, Differential-pulse voltammetry, Nafion

1. INTRODUCTION

Nebivolol (NBV) is a recently approved beta(1)-selective blocker drug for the treatment of hypertension. According to the World Health Organization, hypertension is one of the leading causes of premature death worldwide. Hypertension increases the risk for a variety of cardiovascular diseases, including stroke, coronary artery disease, heart failure, atrial fibrillation and peripheral vascular disease [1]. NBV has additional mechanism besides beta-blocking effects that contribute to its unique pharmacologic profile. It has an endothelium dependent vasodilator property mediated via L-arginine-nitric oxide pathway [2]. Nitric oxide also inhibits platelet and leucocyte adhesion to vascular

endothelium, prevents smooth muscle hyperplasia following vascular injury and scavenging superoxide anion activity.

The development of sensitive analytical methods for determination of NBV is highly required due to the important role of the drug in treatment of chronic diseases. In addition, an official method for its quantification in bulk form and pharmaceutical formulations has not been approved in any pharmacopoeia. Several analytical methods were reported for drug quantification in biological samples and pharmaceuticals including LC-MS methods [3,4], HPLC methods with UV detection [5-7], high performance thin layer chromatography [8], spectrophotometry [9] and spectrofluorimetry [10] Among these methods, electrochemical techniques usually provide greater sensitivity, in addition to its other outstanding features such as rapid response, low cost, easy operation, short analysis time and excellent potential for miniaturization and portable equipment construction [11, 12].

At present, in the literature only three methods were published on the voltammetric behaviour of NBV. The poorly defined voltammetric response was obtained on an electrode modified with undoped silver oxide nanoparticles using two-electrode system [13]. The proposed method was not fully validated and it was not applied to real samples of bulk drug or its pharmaceutical dosage forms. We used unmodified boron-doped diamond electrode for rapid square-wave voltammetric determination of NBV [14], but the working range of the method is suitable for quantification of NBV in its pharmaceutical dosage forms. Very recently, graphene nanocomposite modified electrode was employed for determination of NBV at relatively high potential of 1.33 V versus Ag/AgCl [15].

One of the most widely used nanostructure materials for electrode surface modification is carbon nanotubes (CNTs) [16]. The CNTs are very attractive in the field of sensor development due to their advantageous properties for electroanalysis, such as enhanced electronic properties, high electrical conductivity, excellent chemical stability and high surface-to-volume ratio [17]. Therefore, the CNTs-based sensors generally have higher sensitivities and lower detection limits than traditional carbon electrodes [18]. Also, CNTs have the ability to support metal nanoparticles (NPs). The combination of metal NPs with CNTs can provide a promising way for fabrication of novel sensors with significantly improved performance [19]. Metal exhibits very important physical, optical and electrocatalytic properties that can be improved by mixing with CNTs without losing any of the electronic properties of CNTs. There are several methods of preparing metal NPs-CNT nanocomposites including electrochemical deposition method, electroless deposition, dispersion of metal NPs on the functionalized CNTs and physical methods. For successful external decoration of CNTs, metal NPs could be obtained from bulk metals or the interaction of CNTs with already prepared suspensions of metal NPs [20, 21].

In recent years, the use of zirconium dioxide nanoparticles (ZrO_2 NPs) is rapidly growing in biomedical fields. They are widely used as drug delivery carriers for controlled release of medicines, as gene delivery vehicles with target specificity for some tissues, biocompatible matrix for protein immobilization as well as in orthopedics for improving the properties of traditional bone cements [22]. ZrO_2 NPs have also been employed for construction of various electrochemical sensors and biosensors due to their large surface area, chemical inertness, thermal stability, mechanical strength and lack of toxicity [23-25]. ZrO_2 is naturally available, environmentally friendly and neutral bioceramic material with good potential to be extensively used in pharmaceutical and medical applications.

In this paper, glassy carbon electrode modified with carbon nanotubes and ZrO_2 nanoparticles (ZrONP-CNT/GCE), obtained by simple one-step modification, was used to provide sensitive method for the voltammetric determination of NBV. Nafion is a useful material to enhance the adsorption of the drug molecules onto the electrode [26, 27] and therefore, the nanomaterials were dispersed in a Nafion matrix to ensure high sensitivity. The developed adsorptive stripping differential-pulse voltammetric (AdDPV) method was used for NBV analysis in serum samples. The modified electrode showed excellent performance for direct measurements of NBV in pharmaceutical dosage forms with good accuracy. The voltammetric data were estimated with those obtained by validated HPLC method.

2. EXPERIMENTAL

2.1 Chemicals

Nebivolol hydrochloride was obtained by the Agency for Medicinal Products and Medical Devices (Zagreb, Croatia). Nibel® tablets (Belupo, Croatia) containing nebivolol hydrochloride, equivalent to 5 mg of NBV, were supplied from a local pharmacy. ZrO_2 nanoparticles (purity 99.95%, crystal phase monoclinic), average particle size 20 nm, were purchased from US Research Nanomaterials, Inc. (Houston, USA, http://www.us-nano.com). The multi-walled CNTs (>98%, O.D. 6-13 nm, length 2.5-20 μm), Nafion (5 wt % solution in a mixture of lower aliphatic alcohols and water) and propranolol hydrochloride were obtained from Sigma-Aldrich (Steinheim, Germany). Bisoprolol fumarate, carvedilol and atenolol were supplied from Pliva (Zagreb, Croatia). Hydrochlorothiazide was purchased from Fluka (Laramie, USA).

Stock solutions of NBV (1.0×10^{-3} M) for voltammetric measurements were prepared in ultra-pure water and stored at 4 °C. The standard solutions were prepared by appropriate dilution of these stock solutions with the selected electrolyte just before use. The supporting electrolytes were 0.5, 0.1 and 0.01 M H_2SO_4, 0.1 and 0.01 M HCl and Britton-Robinson buffer adjusted to the desired pH with 0.2 M sodium hydroxide solution.

2.2 Equipment and Conditions

Voltammetric measurements were taken at room temperature using a computer-controlled μ-Autolab potentiostat (Eco Chemie, Utrecht, The Netherlands) with GPES 4.9 software. A three-electrode configuration was used with a bare or modified GCE (3-mm diameter, Metrohm, Switzerland) as working electrode, Ag/AgCl (KCl 3M, Metrohm) reference electrode and a platinum counter electrode. Cyclic voltammetry (CV) was carried out from 0.5 to 1.5 V with the scan rate from 10 to 350 mV s^{-1}. Differential pulse voltammetry (DPV) was used for determination of NBV in the potential range from 0.6 to 1.4 V. The accumulation of NBV was carried out at 0 V with a 240 s step using a computer-controlled stirrer. The pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1} were used. After each measurement, the modified electrode was refreshed in blank electrolyte by one CV scan from 0.5 to 1.4 V until the peak of NBV disappeared completely.

The structure and morphology of nanocomposite was characterized by a field emission scanning electron microscope Jeol JSM-7000F coupled with an energy dispersive X-ray spectroscopy (Jeol Ltd., Tokyo, Japan). HPLC analysis were performed using an Agilent 1100 Series LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector, controlled by ChemStation software.

2.3 Preparation of the ZrONP-CNT/GCE

Before modification, the working electrode was polished with 0.05 μm alumina slurry, rinsed with water and then cleaned ultrasonically for 30 s in water and dried. The suspension (1 mg/mL) was prepared by dispersing the ZrO_2 NPs and functionalized CNTs in 0.5% Nafion ethanol solution to obtain mixture containing a ratio in weight of 1:1 (ZrO_2 NPs-CNTs) under ultrasonic stirring for 2 h prior to use. The carboxylic acid functionalized CNTs were prepared according to the previously described method [28]. The GCE was modified by casting 5 μL of suspension on an electrode surface and dried at room temperature. To obtain a stable cyclic voltammogram and strength adhesion to the electrode surface, the ZrONP-CNT/GCE was scanned prior to the first measurement by two successive cyclic voltammetric sweeps between 0 and 1.5 V at 100 mV s^{-1} in a blank solution of 0.1 M H_2SO_4. To measure the electroactive surface area of the ZrONP-CNT/GCE, the cyclic voltammograms of 1.0×10^{-3} M $K_3Fe(CN)_6$ as redox probe were recorded. According to the Randles-Sevcik equation the electroactive area was calculated to be 0.311 cm^2, nearly 5.5 times greater than GCE [16]. For comparison of electrochemical performance, the ZrO_2 modified GCE (ZrONP/GCE), the carbon nanotubes modified GCE (CNT/GCE) and the Nafion modified GCE (Naf/GCE) were also prepared in the same way as described but without the addition of CNTs, the ZrO_2 NPs and both nanomaterials, respectively.

2.4 Application of the ZrONP-CNT/GCE

To prepare the sample of NBV dosage forms, a quantity of finely ground tablets equivalent to 4.4 mg of NBV was dispersed into a calibrated 10.0 mL flask. The mixture was sonicated for 10 min. The mixture was filtered through 0.45 μm Acrodisc GHP filters (Gelman, Ann Arbor, USA) and was diluted with supporting electrolyte to prepare the solutions in certain concentrations. Solutions were analysed by direct DPV measurements. The content of NBV in the commercial pharmaceutical products was determined by standard addition method. For recovery studies, aliquots of the NBV standard solutions were added to samples prepared from tablets. The NBV determination by HPLC was carried out according to procedure described earlier [14].

Serum samples were collected from healthy volunteers and stored at -20°C until assay. Samples were fortified with aliquot volume of NBV standard solution to achieve concentrations (1.0×10^{-7} M) found in serum after treatment with recommended dose of 10 mg [29]. A 500 μL aliquot of serum sample containing drug was vortexed with acetonitrile (1:1) for 60 s and than centrifuged for 6 min at 6000 rpm. The suitable volumes of supernatant were transferred into the volumetric flask and diluted

with supporting electrolyte before AdDPV measurements. Analysis of human serum samples were performed using the standard addition method.

3. RESULTS AND DISCUSSION

3.1 Morphological characterization of ZrO_2 NPs-CNTs nanocomposite

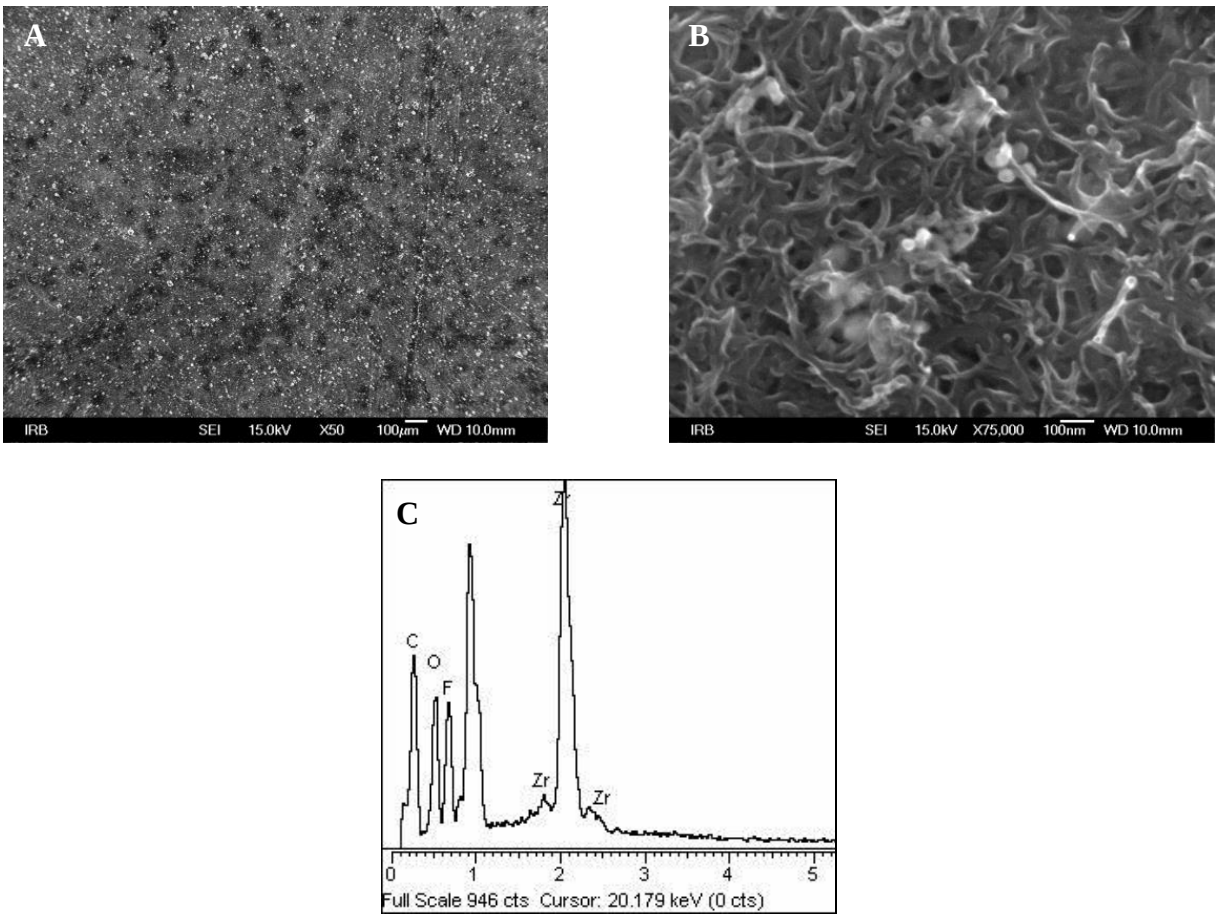

Figure 1. SEM images of the ZrONP-CNT/GCE with different magnifications (A, scale at 100 μm and B, scale at 100 nm) and its corresponding EDS spectra (C).

The surface morphology of the ZrONP-CNT/GCE was examined by a field emission scanning electron microscopy (FE-SEM). As shown in Fig. 1a, low-magnification FE-SEM image indicates that homogeneous film is very uniformly deposited onto the whole surface. The compact coating reveals ZrO_2 NPs observed as white dots well dispersed in CNTs-Nafion matrix. Generally, coating was well adherent to the substrate. The Fig. 1b displays the same surface at high magnification. The unique structure of CNTs with tubes placed in the bundle reveals a large surface area of the coating with ZrO_2 NPs entrapped into the matrix. The ZrO_2 NPs are in uniform spherical shape with average diameter of about 20 nm.

The structure characterization of the ZrO_2 NPs-CNTs nanocomposite was examined using the energy dispersive X-ray spectroscopic (EDS) pattern (Fig. 1c). EDS analysis shows presence of zirconium at 2.10 keV and oxygen at 0.54 keV. The EDS pattern presents carbon peak at 0.26 keV attributed to the carbon nanotubes. The oxygen signal detected also originates from carboxyl and hydroxyl groups introduced onto the CNT surfaces in performed functionalisation procedure [16]. The fluor detected at 0.66 keV is present from Nafion. The peak corresponding to the copper appears at 0.90 keV and comes from the supporting copper grid. The composition of nanomaterial on electrode surface was calculated using the EDS measurement and presented 34.86% of carbon, 18.10% of oxygen, 16.95% of fluor and 30.12% of zirconium.

3.2 Electrochemical properties of NBV on ZrONP-CNT/GCE

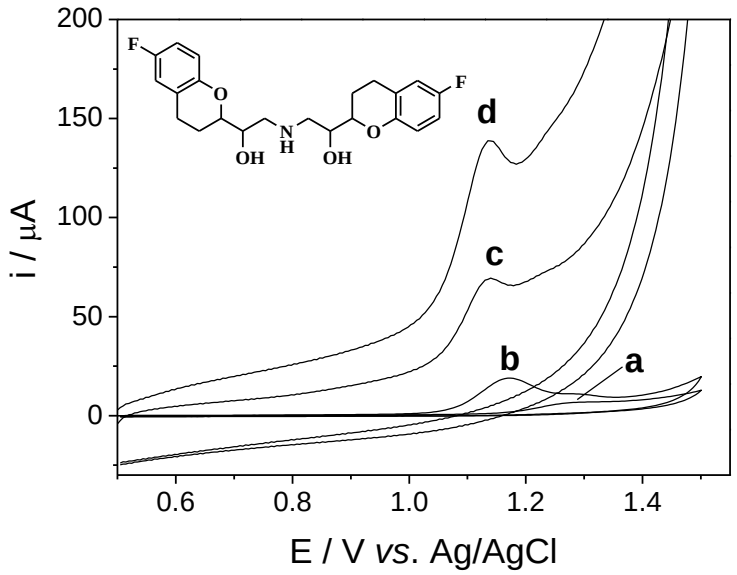

Figure 2. Cyclic voltammograms of NBV (1.0×10^{-4} M) in 0.1 M H_2SO_4 recorded on different electrode surfaces: bare GCE (a), ZrO/GCE (b), CNT/GCE (c) and ZrONP-CNT/GCE (d). Scan rate: 100 mVs^{-1}. Inset: Chemical structure of NBV.

As can be seen in Fig 2, NBV exhibits a weak irreversible voltammetric response (5.6 μA) at 1.28 V using a bare GCE. When the GCE surface is modified with ZrO_2 NPs in Nafion matrix, the response is increased with a current of 18.1 μA. The oxidation peak potential is shifted to less positive values by 110 mV. To enhance sensitivity towards drug molecule, the GCE functionalized with CNTs was also tested under the same condition [16]. After modification of electrode surface, the oxidation peak current intensity on the CNT/GCE is about 7.8-fold higher than that on the bare GCE. Finally, the ZrONP-CNT/GCE reveals a remarkable increase of the voltammetric response of NBV obtained from the combined effects of CNTs and ZrO_2 NPs. Compared with the response obtained at bare GCE, the oxidation peak current is increased up to 88.7 μA at the ZrONP-CNT/GCE with a well-defined response at 1.13 V shifted by 152 mV to less positive potential. This finding is due to increased

electroactive surface area of the ZrO_2 NPs-CNTs nanocomposite as well as the the electronic conductivity and electrocatalytic activity of CNTs modifying layer [16]. Nafion polymer was used to fix the nanomaterials tightly onto GCE [30]. Additionally, the sulfonate groups in Nafion have ability to attract positively charged NBV prior to quantification in order to increase sensitivity.

The effect of scan rate on the oxidative peak currents at the ZrONP-CNT/GCE was also evaluated. In the range of 10 - 350 mV s^{-1} a linear relationship between the peak current and the scan rate was obtained. This result indicates adsorption-controlled process at the ZrONP-CNT/GCE. The dependence of the peak potential is linear with logarithm of the scan rate with a slope of 82.6 mV/decade, indicating αn = 0.72. Using the calculated value of the charge transfer coefficient (0.40), the number of electrons exchanged was found to be n=1.8.

It is known that alcohols are oxidised in many cases in $2e^-/2H^+$ process to ketones as stable intermediates [31]. The voltammetric response observed at ZrONP-CNT/GCE corresponds to the oxidation of one of the secondary hydroxyl groups to the corresponding C=O group in drug molecule (inset of Fig. 2). The same mechanism was proposed for electrooxidation of NBV at boron-doped diamond electrode [14] and graphene nanocomposite modified electrode [15] as well as for other beta-blocker drugs such as propranolol [32] and atenolol whose oxidation product was isolated and characterized by ^{1}H NMR [33].

3.3 Optimisation of ZrONP-CNT/GCE preparation

The relationship between voltammetric signal of NBV at the ZrONP-CNT/GCE and different concentration ratios of ZrO_2 NPs : CNTs (1:1, 2:1) and Nafion concentration (0.3-0.7% v/v) in suspension for surface modification was studied by DPV. The most favorable peak current was achieved at concentration ratio of 1:1 (ZrO_2 NPs : CNTs) with the ZrO_2 NPs concentration of 1 mg mL^{-1} in 0.5% Nafion ethanol solution. The peak current decreased with the increase of ZrO_2 NPs applied on the electrode surface over the concentration of 1 mg mL^{-1}. The decrease of peak current at higher amount of ZrO_2 NPs may be due to lower adhesion between the nanocomposite film and the electrode surface as well as the agglomeration of the nanoparticles [34]. The 0.5% Nafion concentration was the optimized loading of nanomaterials. The film became thicker at higher concentrations hindering the conductivity of nanomaterials doped in polymer.

To obtain a much more sensitive peak current for analytical studies, the variation of NBV current response was examined at the ZrONP-CNT/GCE in different supporting electrolytes, such as sulfuric acid, hydrochloric acid and Britton-Robinson buffer (pH 2-6) [28]. The comparison of voltammograms indicates that NBV exhibits higher peak current at lower pH values. The NBV (pKa=8.9) is completely protonated on the nitrogen atom in acidic media and therefore, more effectively attracted to the modified electrode surface. Analysing the current response, 0.1 M sulfuric acid was chosen as the optimum supporting electrolyte for subsequent analytical measurements.

3.4 Adsorption properties of NBV and analytical performance

Since CV detected some adsorption of NBV at the ZrONP-CNT/GCE, the possibility of analyte preconcentration at electrode surface before DPV measurements was investigated. The differential pulse voltammograms without accumulation and after a 240 s accumulation step at the ZrONP-CNT/GCE are shown in Fig. 3. The improved sensitivity is achieved due to adsorption of NBV through electrostatic interaction with sulfonate group in Nafion matrix [30]. To maximise the DPV signal, the influence of accumulation potential and preconcentration time was estimated in solution containing NBV at 5×10^{-7} M concentration. A maximum enhancement of the voltammetric response was observed at accumulation potential of 0 V. The peak current magnified linearly up to 300 s preconcentration time (inset of Fig. 3), however to achieve the highest sensitivity in shorter analysis time, the value of 240 s was taken as the optimum for NBV quantification.

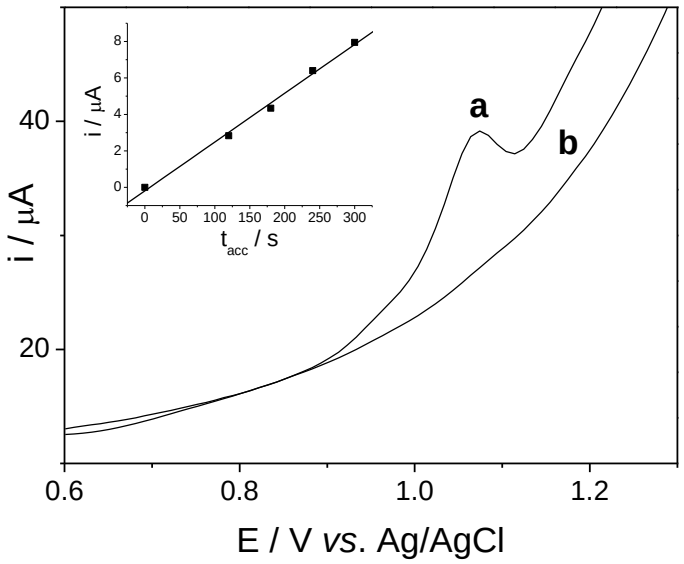

Figure 3. Differential-pulse voltammograms of NBV (5.0×10^{-7} M) at the ZrONP-CNT/GCE in 0.1 M H_2SO_4 after preconcentration time of 240 s (a) and without preconcentration (b). DPV settings: pulse amplitude of 50 mV, pulse width of 75 ms and scan rate of 20 mV s^{-1}, E_{acc} = 0 V. Inset: Influence of the preconcentration time on the NBV oxidation peak current.

Table 1. Analytical parameters for the calibration curves of nebivolol determination using ZrONP-CNT/GCE

Parameter	**DPV**	**AdDPV**
Linearity range (M)	$1.5 \times 10^{-6} – 1.0 \times 10^{-4}$	$1.0 \times 10^{-7} – 6.0 \times 10^{-6}$
Slope (μA M^{-1})	4.36×10^{5}	9.29×10^{6}
Intercept (μA)	-0.37	0.79
$S_{x/y}$	16.21	16.85
SD of slope (S_b)	1.39×10^{4}	2.01×10^{5}
SD of intercept (S_a)	0.072	0.058
Correlation coefficient	0.996	0.998
Limit of detection (M)	5.5×10^{-7}	1.8×10^{-8}
Limit of quantitation (M)	1.6×10^{-6}	6.2×10^{-8}

The electrocatalytic peak current of NBV obtained at 1.05 V using the ZrONP-CNT/GCE was employed for determination of drug in aqueous solution. In the case of a DPV scan without preconcentration, the oxidation peak showed a linear response in the concentration range of 1.5×10^{-6} – 1.0×10^{-4} M. By using drug accumulation at the ZrONP-CNT/GCE prior to DPV measurement (Fig. 4), the current response after 240 s accumulation showed linear relationship in the range of 1.0×10^{-7} – 6.0×10^{-6} M (inset of Fig. 4). The analytical parameters are given in Table 1. The detection limit (LOD) and the quantification limit (LOQ) were estimated as LOD = 3s/b and LOQ = 10s/b, where s is the standard deviation of the intercept and b is the slope of the calibration curve [35].

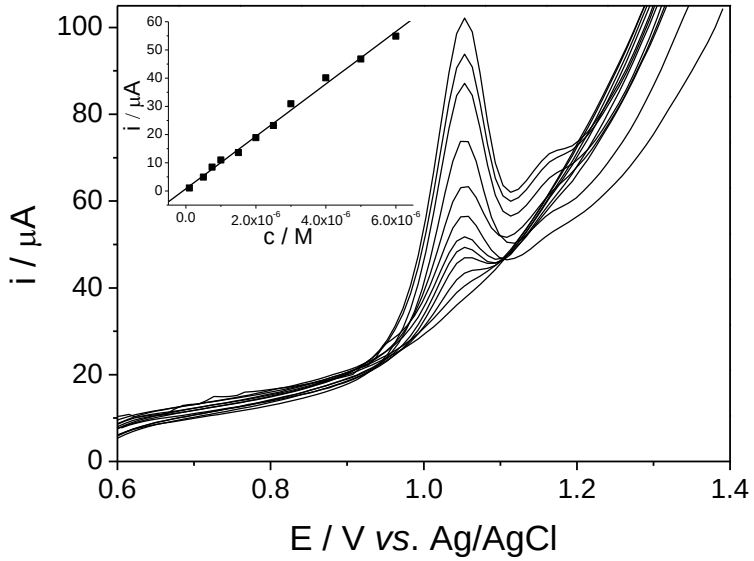

Figure 4. Adsorptive stripping differential-pulse voltammograms of NBV at ZrONP-CNT/GCE in 0.1 M H_2SO_4 recorded at different concentrations (1×10^{-7} – 6×10^{-6} M) after preconcentration time of 240 s with corresponding background recording. DPV settings same as in Fig. 3. Inset: calibration graph for NBV quantification.

3.5 Selectivity, precision and stability of ZrONP-CNT/GCE

The effect of different possible interfering species in the analysis of NBV was studied in the mixed solutions of these species with 2.5×10^{-6} M NBV using AdDPV method. The tolerance limit was calculated as molar ratio of NBV/interference which gave an error less than ±5% in the determination of the drug [36]. It was found that some ions such as Na^+, K^+, Cl^-, HPO_4^{2-} had no influence on NBV response at concentration of about 1000-fold of NBV. The experiments also revealed that NBV response did not change after adding 500-fold of ascorbic acid, lactose and citric acid as well as 400-fold of glucose. In the presence of uric acid, new well-defined oxidation peak was registered at the potential +0.49 V. However, the results showed that 100-fold concentration of uric acid did not cause an increase in the NBV current as well as dopamine present in equal concentration. However, the interference effect was observed with equal concentrations of other beta-blockers like atenolol, carvedilol, propranolol and bisoprolol. In the presence of propranolol and carvedilol, new

oxidation peaks were found in the voltammograms (+0.75 V for propranolol and +0.96 for carvedilol) with a significant influence on the distortion of the NBV response (-10.1% and -38.9%, respectively). On the other hand, atenolol and bisoprolol did not display well-developed voltammetric response in investigated potential range; however, these substances caused a positive error (+9.3% and +25.4%, respectively) in the NBV current. Furthermore, the interference studies showed that the equal concentration of hydrochlorothiazide caused an increase of 46.4% in the NBV current response. Hydrochlorothiazide is used for the treatment of hypertension, separately or together with NBV in a combined pharmaceutical formulation. Its oxidation potential (+1.02 V) is very close to that of NBV, resulting in overlapping voltammetric responses (Fig. 5).

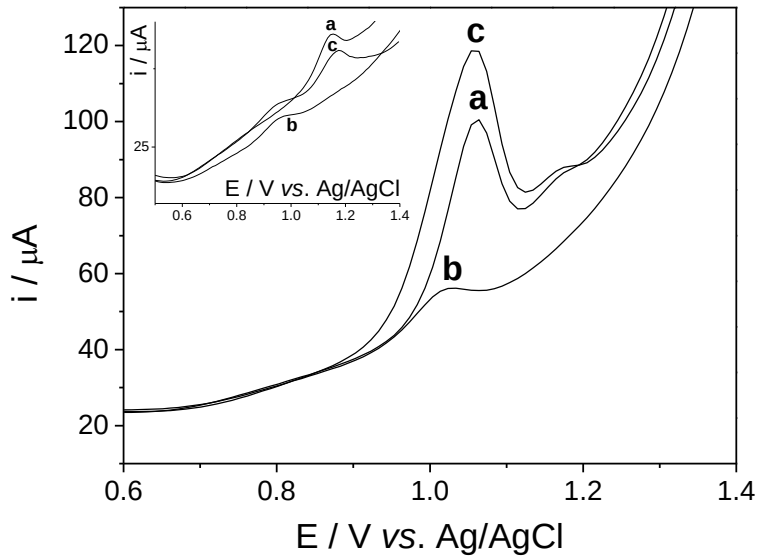

Figure 5. Comparison of voltammetric responses of NBV (line a), hydrochlorothiazide (line b) and their mixture (1:1) (line c) recorded at ZrONP-CNT/GCE in 0.1 M H_2SO_4. DPV settings same as in Fig. 3. Inset: voltammetric responses recorded in BR buffer pH 4.

To obtain the differentiation of the responses, the influence of supporting electrolyte pH value (0.1 M sulfuric acid and BR buffer pH 4) on peak potentials was studied on a mixture containing both drugs. With the increase of pH, the peak potential of hydrochlorothiazide was shifted to less positive value (inset of Fig. 5). Unfortunately, the potential difference obtained at the ZrONP-CNT/GCE was not large enough to overcome this problem. Therefore, the ZrONP-CNT/GCE cannot be applied to simultaneous analysis of NBV and hydrochlorothiazide as well as studied beta-blockers.

For method validation, the precisions of the electrode response were evaluated by adsorptive stripping DPV measurements of 2.5×10^{-6} M NBV solutions. Unchanged oxidation peak potential and the RSD value of 1.1% for peak current (mean i_p = 23.2 μA) were estimated for intra-day measurements with the same modified electrode. The inter-day precision of the electrode response was tested by three replicate measurements over three days [16], each time using a freshly prepared ZrONP-CNT/GCE. The RSD value of the peak current was 2.8%, while the RSD of peak potential for was 0.4%, indicating excellent fabrication reproducibility.

The stability of the ZrONP-CNT/GCE was examined by recording the voltammetric response over the course of two weeks. The modified electrode was kept at room temperature between experiments. The peak potential was unchanged, while the drug current response decreased after a 10-day use by 4.1%. After this period the signal decreased progressively. To explore a reusable application of electrochemical sensor the renewability of the ZrONP-CNT/GCE was examined considering the adsorption of NBV or its oxidation product on the modified surface. A simple electrochemical approach to clean the used sensor was used by running CV between 0.5 and 1.4 V in blank supporting electrolyte. The voltammetric responses obtained at regenerated ZrONP-CNT/GCE after CV scan were compared with the ones obtained during its first-time use. The regenerated sensor exhibited similar responses (recovery of 99.1±2.5% based on three measurements) indicating that the adsorbed molecules were successfully removed through the cleaning process.

3.6 Analytical applications

The ZrONP-CNT/GCE in combination with the DPV was applied for direct measurement of NBV in pharmaceutical formulations using the standard addition method in order to eliminate matrix effects. The assay showed that drug content of pharmaceutical product is in good agreement with the declared value (Table 2).

Table 2. Analysis of NBV in pharmaceutical dosage forms by the proposed DPV method at ZrONP-CNT/GCE and HPLC method

	DPV	HPLC
Label value (mg)	5	5
Determined value (mg)[a]	4.92	4.91
Recovery %	98.3	98.2
RSD %	1.15	0.64
Bias %	-1.60	-1.80
Added (M)	1.00×10^{-5}	3.00×10^{-5}
Found (M) [a]	0.98×10^{-5}	2.96×10^{-5}
Recovery %	98.1	98.7
RSD %	1.28	0.87
Bias %	-2.00	-1.33
F [b]	3.21	-
t [b]	0.85	-

[a] n=5

[b] The theoretical values of F and t-test at 95% confidence limit are 6.39 and 2.31, respectively.

The recovery study was carried out by adding known concentration of standard to the formulation solution to define the accuracy of the new method using ZrONP-CNT/GCE. In addition, the results obtained with the voltammetric method were compared with those obtained by developed

reverse-phase HPLC method [14] for NBV (Table 2). The results of the student *t*-test and variance ratio *F*-test show that there are no significant differences between the methods regarding the accuracy and precision. This finding revealed that the ZrONP-CNT/GCE can be used as a reliable and inexpensive electrochemical platform for quantification of NBV in pharmaceutical preparation. Moreover, the method developed at the ZrONP-CNT/GCE is faster and simpler in comparison with HPLC (the retention time of NBV was 17.6 min) [14].

The serum concentration of NBV administered in therapy of hypertension and cardiovascular diseases, the leading cause of the death in the world, can be monitored only after preconcentration of the drug on the modified electrode surface. To check the potential of ZrONP-CNT/GCE for use in analysis of NBV at trace level, the AdDPV method was applied to drug determination in human serum. NBV is on the list of prohibited substances in certain sports according to list of several anti-doping agencies. Therefore, the ZrONP-CNT/GCE was also tested for screening of drug intake or abuse. The standard addition method was used for the recovery studies in serum samples. The results are presented in Table 3. From mean recovery of 100.8±1.7%, it is obvious that the ZrONP-CNT/GCE has potential to determine of NBV in serum samples.

Table 3. Quantification of NBV in serum samples at ZrONP-CNT/GCE by adsorptive stripping DPV

Added 10^7 c (M)	Found 10^6 c (M)	Recovery (%)	RSD[a] (%)
1.50	1.51	100.67	1.99
2.50	2.52	100.80	2.21
5.00	5.09	101.87	2.16
7.50	7.49	99.87	0.61

[a] average of three measurements

3.7. Comparison of analytical methods

The ZrONP-CNT/GCE was compared with other electrochemical sensors developed for the determination of NBV (Table 4). The higher sensitivity was obtained at the ZrONP-CNT/GCE for measurement of NBV and wider linear range was achieved allowing the use of method for both applications, pharmaceutical products and biological samples. The LOD value obtained at the ZrONP-CNT/GCE using adsorptive stripping DPV is in lower concentration range than that obtained at other electrodes, except for the electrode modified with undoped silver oxide nanoparticles [12]. However, it is unclear whether the current response at this electrode has changed with concentration, and the method was not fully validated. The method developed using unmodified boron-doped diamond electrode is faster but obtained sensitivity is lower compared to the ZrONP-CNT/GCE.

In relation to the other developed analytical methods for determination of NBV, the linearity obtained at the ZrONP-CNT/GCE is in a lower concentration range compared to the previously reported HPLC methods with UV detection [6,9]. The LOQ value obtained by AdDPV is better than those found with HPLC using UV detector [6,9], high performance thin layer chromatography [8],

spectrophotometry [9] and comparable with spectrofluorimetry [10]. The LOD value is higher than LODs reported for LC-MS method [4,5], but this technique is not widely available.

Table 4. Comparison of various voltammetric methods for the determination of NBV with the present work

Technique	Electrode	Concentration range (M)	Sensitivity ($\mu A\ M^{-1}$)	LOD (M)	Reference
I-V[a]	Undoped AgO NPs/GCE	$5.5 \times 10^{-9} - 9.9 \times 10^{-5}$	3.48×10^{3}	0.9×10^{-9}	12
SWV[b]	BDDE	$2.5 \times 10^{-7} - 1.5 \times 10^{-5}$	3.46×10^{5}	3.2×10^{-8}	13
AdDPV	Graphene-Nafion/GCE	$5.0 \times 10^{-7} - 2.4 \times 10^{-5}$	4.52×10^{5}	4.6×10^{-8}	14
DPV	ZrONP-CNT/GCE	$1.5 \times 10^{-6} - 1.0 \times 10^{-4}$	4.36×10^{5}	5.5×10^{-7}	This work
AdDPV	ZrONP-CNT/GCE	$1.0 \times 10^{-7} - 6.0 \times 10^{-6}$	9.29×10^{6}	1.8×10^{-8}	This work

[a] I-V measurement in two electrode system

[b] Square-wave voltammetry

[c] Boron-doped diamond electrode

4. CONCLUSION

A simple modification of a GCE with ZrO_2 NPs and carboxylic acid functionalized multi-walled CNTs was examined and elaborated. Nanocomposite film displayed high electrocatalytic activity to oxidation of NBV. DPV and AdDPV were used to investigate the sensitivity of ZrONP-CNT/GCE to drug molecule oxidized at 1.03 V. The modified electrode showed a wide linear range for NBV with high sensitivity. The ZrONP-CNT/GCE is able to detect NBV in serum samples. This modified electrode can be used with short response time for direct measurements of NBV in pharmaceutical dosage forms with satisfactory results in comparison with HPLC method.

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