A highly Sensitive Modified Glassy Carbon Electrode with Carboxylated Multi-walled Carbon Nanotubes/Nafion Nano composite for Efficient and Cheap Voltammetric Sensing of Dianabol Steroid in Biological Fluid

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Abstract

The extraordinary prerequisite for analysis of anabolic steroid namely dianabol (DB) has inspired towards the development of cost effective and high-performance sensing probe. Thus, a simple and robust electrochemical sensor (c- MWCNTs-Nafion®/GCE) for dianabol (DB) a widely used steroid was developed using a glassy carbon electrode (GCE) modified with functionalized carboxylated multi-walled carbon nanotubes (c-MWCNT) and Nafion®. At pH 7-8, differential pulse-cathodic stripping voltammetry (DP-CSV) displayed two cathodic peak at -0.85 and -1.35 V that varied linearly over a wide range (9.0×10^{-9} ($2.7 \mu\text{g L}^{-1}$) - 9.0×10^{-6} ($2.7 \times 10^3 \mu\text{g L}^{-1}$) mol L⁻¹) and 2.9×10^{-6} ($8.7 \times 10^2 \mu\text{g L}^{-1}$) - 8.0×10^{-5} ($2.4 \times 10^4 \mu\text{g L}^{-1}$) mol L⁻¹) of DB concentrations, respectively. The low limits of detection and quantification at peak I (-0.85 V) were 2.7×10^{-9} ($8.1 \times 10^{-1} \text{ ng mL}^{-1}$) and 9.0×10^{-9} (2.7 ng mL^{-1}) mol L⁻¹, respectively. The repeatability and reproducibility displayed relative standard deviations lower than 5%. The method was applied for DB analysis in human urine and subsequently compared with standard HPLC method. Interference of common metabolites in biological fluids samples to DB sensing was insignificant. This method has distinctive advantages e.g. precise, short analytical time, sensitive, economical, reproducible and miniaturized sample preparation for DB analysis in biological samples of human origin.

Keywords: Anabolic steroid; Biological fluids; Differential pulse-cathodic stripping voltammetry; Modified glassy carbon electrode; Method validation.

1. Introduction

The use of drugs to improve human performance is a major in sport activities for many years. The exogenous anabolic steroid dianabol chemically namely methandrostenolone or 17 β -hydroxy-17methylandrosta-1,4-dien-3-one) (Electronic supplementary information

(ESI-1) has been widely used due to its great ability to stimulate protein synthesis and improve compensatory adaptation in the humans.¹⁻³ However, use of such drugs pose a great number of opposing properties like disturbance of the propagative system^{4,5}, liver/kidney damage⁶, reduced fertility⁵, hypertension as well as atherosclerosis.⁷⁻⁹ Therefore, determination of hormonal drug in biological fluid is very crucial to monitor the health of human being. On the other hand, their subsequently release from biological fluids (such as urine) into the environment have raised great deal of concern for environmentalists due to emerging issue of water pollution. Hence, it is duly important to establish novel method for rapid detection and precise quantification of trace levels of such class of steroids.^{10, 11}

Numerous chromatographic methods¹²⁻¹⁵, **magnetic MOF-101 derivative coupled with high-performance liquid chromatography (HPLC)**¹⁶ and thermal desorption atmospheric pressure photoionization mass spectrometry¹⁷ are known for analysis of steroids and other biomolecules e.g. bovine hemoglobin¹⁸ in complex matrices. Most of the chromatographic methods are time consuming, require sample pre-treatment and are not appropriate for measuring at trace level concentrations.¹⁹⁻²¹ This issue was overcome through inclusion of solid phase microextraction step²² or using highly complex yet very sensitive UHPLC-MS/MS.²² The main drawbacks of most of these methods are the high cost of the GC instruments, complication, enrichment step, and the need of well skilled personnel for their proper operation. Further, in GC techniques, derivatization is highly required for making the compounds volatile when compared to HPLC, solid phase extraction, hydrolysis and liquid–liquid extraction.²²⁻²⁵ An aptamer based approach was proposed by Jauset-Rubio et al.²⁶, where the developed probes could simultaneously identify different steroids such as estradiol, progesterone, and testosterone using microtiter plate assay.

Recently, the use of electrochemical sensors for analysis of steroids e.g. methandienone and other various analytes (including steroids/drugs)^{27,28} in food and water has become one of the prime importance for analytical chemists.²⁹⁻³¹ A series of nanomaterials e.g. carbon nanotubes as novel nanometer-sized with unique properties, TiO₂, SiO₂, Ag, Au, Ir₂O₃, graphene and graphene oxide, Bi₂O₃ etc., have been widely used in constructing electrochemical sensors or biosensors.³²⁻³⁴ When compared to chromatographic techniques, they are easy to fabricate, harbor superior stability and do not require any form of pre-treatments prior to analysis.^{35, 36} Based on preliminary study on electrochemical detection of DB steroid, DP-CSV technique was chosen for DB analysis, because it is easy to use, inexpensive and short analysis time. Moreover, DP-CSV technique has a low capacitive current combined with a great discrimination of faradaic current that can improve the sensitivity of the present approach towards DB analysis.³⁷ Thus, in the current study we have focused on: i).authorizing the efficiency, selectivity as well as stability of the modified GCE towards target steroid; ii) assigning the most probable electrochemical mechanism of the electrode couple and finally iii) demonstrating the analytical utility of the established DP-CSV method for detection of DB in biological fluids (urine) samples.

2. Experimental

2.1 Reagents and materials

Analytical reagent grade (A.R) chemicals were used as received. Glassware's were washed with hot soaps, 50% HCl (Analar), HNO₃ (2.0 mol L⁻¹), re-cleaned with HCl (0.5 mol L⁻¹), rinsed with deionized water and acetone, and finally dried at 120 °C. Low-density polyethylene (LDPE) bottles, Nalgene were used for storing the aliquots. Dianabol (17β-Hydroxy-17methylandrosta-1, 4-dien-3-one) (**ESI-1**) with 99% (HPLC) purity was obtained

from Sigma-Aldrich (Steinheim, Germany). Stock solution ($1.0 \times 10^{-2} \text{ mol L}^{-1}$) of dianabol (DB) was prepared in deionized (DI) water in the presence of few drops of ethanol and completed to the mark with ultra-pure water. More diluted working solutions (2.0×10^{-9} – $1.0 \times 10^{-4} \text{ mol L}^{-1}$) of DB were also prepared and stored in low-density polyethylene (LDPE) bottles. Multiwalled carbon nanotubes (MWCNTs) (dimensions: length = 0.5–2.0 μm , ID = 5–40 nm, OD = 40–70 nm, C 95%) were purchased from Aldrich, USA. Britton-Robinson buffer of varying pH (2.0 to 11.0) was prepared as reported.³⁸ Series of phosphate buffer solutions (pH 7.0–9.0) were prepared by mixing of KH_2PO_4 ($1.0 \times 10^{-1} \text{ mol L}^{-1}$) and K_2HPO_4 ($1.0 \times 10^{-1} \text{ mol L}^{-1}$) in water and adjusting the solution pH to the required value with H_3PO_4 ($1.0 \times 10^{-1} \text{ mol L}^{-1}$) and/or NaOH ($1.0 \times 10^{-1} \text{ mol L}^{-1}$). HEPES buffer (pH 6.0–8.0; $3.0 \times 10^{-1} \text{ mol L}^{-1}$) was obtained from Sigma-Aldrich were also prepared by dissolving the required weight in 10 mL of deionized water and served as supporting electrolyte. Nafion (4% w/v) in ethanol was obtained from Sigma that was further diluted (0.08–1% v/v) in ethanol.

2.2 Instrumentation

The differential pulse and cyclic voltammetric measurements of the steroid DB were performed at RT on Metrohm 757 VA trace analyzer and 747 VA stand (Basel, Switzerland). 10 mL voltammetric cell (Metrohm), including bare GCE (d=2.0 mm) or Nafion/c-MWCNTs grafted GCE) as working electrodes, Ag/AgCl ($3.0 \text{ mol L}^{-1} \text{ KCl}$) and platinum wire (BAS model MW-1032) as standard and auxiliary electrodes, respectively. A JEOL-JSM6301-F (Peabody, MA, USA) scanning electron microscopy (SEM) was used for recording the surface topography of GCE and Nafion/c-MWCNTs/GCE. Volac digital micro pipettes 10–1000 μL were used to prepare the solutions. The cathodic peak current was recorded using “tangent fit method”.³⁹ A Milli-Q Plus system (Millipore, Bedford, MA,

USA) and Martini pH meter (Model Mi 150) were used for delivering the deionized (DI) water and measuring the solutions pH, respectively.

2.3 Recommended procedures for DB determination

The procedures for the preparation of Nafion/c-MWCNTs nanocomposites and fabrication of Nafion /c-MWCNTs nanocomposite film coated GCE were described in more details in **ESI-2**. The DP-CSV procedures were performed at bare and/or Nafion/c-MWCNT coated GCE as follow: A 10.0 mL of phosphate buffer solution (pH 7.4) was transferred to the electrochemical cell, stirred, and purged with N₂ gas for 15 min. The DP-CSV of the supporting electrolyte was recorded by applying negative potential from -0.8 to -1.5 V vs Ag/AgCl reference electrode at the optimized parameters of accumulation potential (0.0 V) and time (180 s); scan rate (60 mV s⁻¹) and pulse amplitude (70 mV) at bare GCE and modified Nafion/c-MWCNT GCE after 10 s equilibration time. At the optimized parameters, the DP-CS voltammograms were then recorded at known concentrations (2.0×10^{-9} – 1.0×10^{-4} mol L⁻¹) of DB at the potential -0.8 to -1.5 V. Similarly, the impact of scan rate on the cathodic peak current and potential of DB (1.0×10^{-4} mol L⁻¹) at optimum pH 7.4 was studied at bare and Nafion/c-MWCNT modified GCE at the optimized parameters. Next to each measurement, CVs were performed in a blank solution for 20 cycles to regenerate the electrode surface.

2.4 Analytical applications

A 1.0 mL of DB-free urine specimen from healthy donor (male, age 30 year) and 1.0 mL phosphate buffer (pH 7.4) were placed into the voltammetric cell and diluted with DI water to 10.0 mL. The DP-CSVs were recorded in the absence and presence of various known concentrations of DB. The resulting DP-CSV cathodic response was used for construction

of the standard addition plot by plotting DB concentration vs. the corresponding cathodic peak current. The standard addition curve was used for computing unknown concentrations of DB and recovery percentage. DB concentration was calculated with the help of the calibration plot employing the following equation (1):

$$[\text{DB}] = b C_s / m \cdot V_x \quad (1)$$

where b and m are intercept and slope of the linear plot, respectively; C_s is the standard DB concentration, V_x is the volume of the aliquot.

3. Results and discussion

3.1 Characterization of the modified Nafion/c-MWCNTs/GCE

The surface morphology of the bare GCE, Nafion/GCE and Nafion/c-MWCNT/GCE nanocomposites modified GCE was studied using SEM with suitable magnification scale (100,000 $\times$). The SEM images of the bare GCE, Nafion/GCE and Nafion/c-MWCNT film grafted onto GCE are illustrated in Figure 1 (A-C), where bare GCE (Figure 1A) showed no morphological aspects on its surface, while Nafion modified GCE displayed a smooth film of Nafion (Figure 1B). Upon grafting Nafion/c-MWCNT film onto GCE, a uniform coating of porous reticular fragments of c-MWCNTs implanted in Nafion matrix was clearly visible. The SEM images of Nafion/c-MWCNT also show many nanotubes twist together and the purity of the treated c-MWCNT is very high since no observable carbon particle impurities were noticed.^{40,41} The exclusive morphology of Nafion/c-MWCNT/GCE (Figure 1C) confirms a large surface area with highly accessible active sites, facilitating electron transfer and stimulating the electrochemical sensing, resulting enhancement of the current signal and consequently highly improves the analysis process of DB. On the other hand, the surface modification on the topography of the modified GCE was further studied by AFM analysis.

Representative AFM images are shown in Figure 1 D. The image revealed significant increase in the surface area of the modified and the available active sites at the Nafion/c-MWCNT/GCE compared to the non-modified GCE.

Please insert Fig. 1 (A-C)

The surface area of bare GCE and Nafion/c-MWCNTs/GCE was determined from the CVs of $K_3[Fe(CN)_6]$ (1.0×10^{-3} mol L⁻¹) in KCl (0.1 mol L⁻¹) solution in suitable potential window (-0.2 to 0.8 V) at various scan rates *versus* Ag/AgCl electrode. The effective surface area of the working electrode for reversible electrode reaction can be calculated using Randles-Sevcik equation (2):⁴¹

$$i_{p,c} = (2.69 \times 10^5) n^{3/2} A C_0 D_R^{1/2} v^{1/2} \quad (2)$$

where, A=surface area of the Nafion/c-MWCNTs/GCE electrode, D_R =diffusion coefficient, v =scan rate, n =number of electron transfer, C_0 =concentration of $K_3[Fe(CN)_6]$. For the electrode couple Fe^{2+}/Fe^{3+} $n=1$ and $D_R=7.60 \times 10^{-6}$ cm²s⁻¹.³⁷ The plots of the cathodic peak current $i_{p,c}$ vs. $v^{1/2}$ for of $K_3[Fe(CN)_6]$ at GCE and Nafion/c MWCNT/GCE were found linear (ESI-3 a, b). Assuming the value of $n=1$ for the electrode couple Fe^{3+}/Fe^{2+} , the consistent surface areas of Nafion/c-MWCNT/GCE and bare GCE as calculated from the slopes of the linear plots (ESI-3 a, b) were 0.158 and 0.036 cm², respectively. These values are quite close to the data reported.⁴²

3.2 Redox behavior of DB at modified GCE

The DP-CSV responses of DB (9.9×10^{-5} mol L⁻¹) at bare GCE and Nafion/c-MWCNTs/GCE, in phosphate buffer of pH 7.4 was recorded (Figure 2). At a bare GCE, weak cathodic peak at $E_{p,a} = -1.26$ V was observed, whereas Nafion/c-MWCNTs/GCE

showed well-defined cathodic peaks at -1.35 V *versus* Ag/AgCl, respectively. Nafion/c-MWCNTs/GCE exhibits enhancement for the cathodic peak current of DB at -1.35V compared to the results obtained with a bare GCE. Moreover, Nafion/c-MWCNTs/GCE shows significantly improved sensitivity for the analyte determination as reported earlier.³²

The cathodic peak current ($i_{p,c}$) at the Nafion/c-MWCNTs/GCE modified electrode was found slower electron transfer than bare GCE. The inorganic ion exchanger Nafion facilitates the attraction of the DB molecules holding a positive charge from the bulk solution. This effect is mostly likely contributed to the observed enhancement of the cathodic peak current signal in the presence of c-MWCNT.^{39,40} Then, due to the low response of DB at bare GCE, it is not suitable for DB sensing under the employed conditions. Thus, in the succeeding study, Nafion/c-MWCNT/GCE sensor was selected as a proper electrochemical sensor for DB analysis.

Please insert Fig. 2

The reduction peak potential of DB at Nafion/c-MWCNTs/GCE was noticed at more negative potential (-1.35 V) compared to bare GCE (-1.26 V), revealing fast electron transfer and magnificent of the electrocatalytic activity of the former electrode towards analyte. An increase in the cathodic peak current of DB at Nafion/c-MWCNT nanocomposite film coated GCE most likely attributed to the considerable increase in the surface area and good conductivity formed by grafting Nafion/c-MWCNT nanocomposites onto the GCE electrode.^{39,40} The observed increase of the electron transfer kinetics at Nafion/c-MWCNTs nanocomposite film coated GCE originates from the high conductivity of the modified GCE and the large surface area available for the intercalation of the DB on the modified GCE. The electrocatalytic activity and electronic conductivity of c-MWCNTs,

in addition to the ionic conductivity and ion-exchange power of Nafion, may also contributed in the observed trend of Nafion/c-MWCNT composites. The segments of Nafion polymer structure have the ability to electrostatically interact with DB molecules from the bulk solution, and improving the analytical sensitivity.⁴³ Combination of Nafion with c-MWCNTs also increased the constancy of the sensor via formation of a tight sensing film onto the electrode surface.⁴⁴

The electrochemical reduction process of DB was studied by recording the cyclic voltammograms at different scan rates (v). In phosphate buffer of pH 7.4, the CVs of DB at various scan rates (20-80 mV s^{-1}) at the modified GCE *versus* Ag/AgCl electrode are represented in **Figure 3**. An ill-defined cathodic peak at peak potential in the range -1.30 to -1.40V was noticed whereas in the reverse scan no anodic peaks were observed in the same potential window (-0.8 to -1.8V).

Please insert Figure 3

The plot of $\log i_{p,c}$ *versus* $\log v$ was found linear (**ESI-4 A**) with the following regression equation (1):

$$\log i_{p,c} (\mu\text{A}) = 0.412 \log v (\text{mV s}^{-1}) - 0.265; R^2 = 0.996 \quad (3)$$

The $E_{p,c}$ *versus* $\log v$ was found also linear (**ESI-4 B**) and can be described by the following regression equation (5):

$$E_{p,c} (\text{V}) = 0.033 \log v (\text{V s}^{-1}) - 1.347; R^2 = 0.995 \quad (4)$$

The calculated slope (0.412) of the linear plot of $E_{p,c}$ *versus* $\log v$ (**ESI-4 B**) was found close to the theoretical value (0.5) for pure diffusion-controlled process.^{43,44} The observed cathodic peaks potential ($E_{p,c}$) were also shifted to less negative values on increasing the scan rate, confirming the irreversible nature of the electrochemical reduction step and the

diffusion of DB from the bulk solution to the Nafion/c-MWCNTs electrode surface.⁴⁴⁻⁴⁶

Typical CVs in the potential range -1.05 to -1.65 V *versus* Ag/AgCl electrode at various scan rates are also shown in Fig. 3. Thus, the present reduction of DB at the modified GCE involves one H⁺/one electron step as described earlier³² as shown in Scheme 1 (ESI-5).

On growing the scan rate, the plot of $i_{p,c}$ *versus* $v^{1/2}$ at Nafion/c-MWNTs/GCE electrode (ESI-6 A) increased in nonlinear form (ESI-6) . Thus, the electrode process is adsorption-controlled features.^{44,45} The values of the i_{pc} of both electrochemical processes were proportional with raising the scan rate supporting the occurrence of an adsorption-controlled reaction which is further expressed by Laviron model.⁴⁷ The current function ($i_{p,c}/v^{1/2}$) decreased on growing the scan rate at -1.35 V for DB (5.0×10^{-5} mol L⁻¹) as demonstrated in ESI.6B showing that the electrochemical reduction process of DB favors the electrochemical-chemical-electrochemical (ECE) type mechanism. The first electrochemical reduction step comprises two consecutive reductions as demonstrated in scheme 1 which appear as separate cathodic peaks (ESI. 5) where the chemical rate constant is 'fast'.^{45,46}

Laviron⁴⁷ model is commonly recognized for an irreversible electrode process by the following expression:

$$E_{p,c} = E^{\circ} + \frac{2.303RT}{\alpha nF} \log \frac{RTK^{\circ}}{\alpha nF} + \frac{2.303RT}{\alpha nF} \log v \quad (5)$$

where, α = electron transfer coefficient, and other symbols have their usual meanings. The value of αn value was computed from the slope of the linear plot of $E_{p,c}$ vs. $\log v$ (ESI-4B).

Considering the slope value =0.033 at T = 298 K, F = 96480 C and R = 8.314 J K⁻¹ mol⁻¹, the value of αn was calculated as 1.8. The corresponding value of α was calculated using the equation (4):^{39, 48}

$$\alpha = 47.7/(E_p - E_{p/2}) \quad (6)$$

where $E_{p/2}$ is the potential at half of the maximum peak current. The value of α was calculated as 0.795, and the number of electrons transferred (n) involved in the reduction process of DB was determined as 2.2. This value is predictable for an irreversible electrode process involving $2H^+ / 2e$ transfer step.^{39, 48} Thus, the electrode process is most likely involved unstable electrogenerated chemical species as one H^+ /one electron step in addition to another defined electrode process involves another one H^+ /one electron step corresponding to observed cathodic peak.

3.3 Optimization of the analytical parameters

The DP-CSV and CVs results of DB at the modified GCE revealed considerable degree of adsorption and sensitivity at the grafted GCE surface. Thus, detailed studies involving the influence of analytical parameters that control performance of the modified Nafion/c-MWCNTs nanocomposite film coated GCE towards developing low-cost and precise procedures for DB determination were discussed below.

The effect of the accumulation time (0.0–90s) on the cathodic peak current of DB at pH 7.4 using DP-CSV was investigated. The data are demonstrated in Figure 4A shows a well-defined cathodic peak was achieved at $E_{p,c} = -1.35$ V and maximum peak current was attained after 60s that leveled off at longer time; most likely due to saturation of the electrode surface with DB. This is a characteristic feature of adsorptive stripping. Thus, in the subsequent work, an accumulation time of 60s was adopted.

The cathodic peak current of DB was also evaluated at different deposition potentials (-1.6 to 0.2 V) vs. Ag/AgCl at 60s accumulation in phosphate buffer pH 7.4. The results are illustrated in Fig. 4B. Maximum peak current at 0.0 V was attained. Thus, in the subsequent

work, an accumulation potential of 0.0V was selected. At potentials less than 0.0 V, the peak current was decreased, reaching to -1.4 V or less, the peak current was completely diminished, indicating that lower potentials do not support the accumulation of DB on the electrode surface.

The effect of DP scan rate ($10\text{--}100\text{ mVs}^{-1}$) on the $i_{p,c}$ of DB ($5.0 \times 10^{-5}\text{ mol L}^{-1}$) determination at -1.35 V was critically studied at the optimized experimental conditions. On growing the scan rate up to 70 mV/s , the current increased and leveled off at higher sweep rate (Figure 4C). However, in the next experiments, a 60 mVs^{-1} scan rate was adopted, since at this sweep rate sharp, symmetric, and well-defined cathodic peak was observed. The DP-CSVs of DB was recorded over a wide range of pulse amplitudes ($0.0\text{--}100\text{ mV}$) at the optimized parameters. A symmetric, sharp, and highest cathodic peak was noticed at 50 mV (Figure 4D). Thus, pulse amplitude of 50 mV was selected in the succeeding study.

Please insert Figure 4 (A-F)

The composition of c-MWCNT in the fabrication of the modified GCE is crucial. The plot of the cathodic peak current at -1.35 V of the DP-CSVs of DB ($1.0 \times 10^{-5}\text{ mol L}^{-1}$) *versus* mass of c-MWCNTs ($2.0\text{--}8.0\text{ mg}$) are illustrated in Figure 4E. On raising the c-MWCNT concentration in the modifying suspension up to 0.06 mg/mL of Nafion/ethanol solution, the $i_{p,c}$ at -1.35V increased, and leveled off at higher c-MWCNTs composition. This profile can be assigned by the charge transfer resistance offered by higher amounts ($>0.06\text{ mg/mL}$) of c-MWCNTs due to the formation of an increasingly thick film, which may hinder the DB diffusion as reported earlier.³⁴ The potential aggregation of c-MWCNT at high c-MWCNT composition may also inhibited the fixation of Nafion/c-MWCNT nanocomposite on the clean bare GCE. The stability of the film grafted onto GCE is consequently decreased.^{39,48}

Thus, 0.06 mg/mL of c-MWCNT in Nafion/ethanol nano composite was adopted for modification of the GCE for use in the successive experiments for DB sensor.

Nafion composition in the nanocomposite is of great importance for modification of the GCE. Thus, impact of Nafion was critically tested by varying its concentration (0.05-0.20% v/v) in ethanol at the optimized content of c-MWCNTs (0.06 mg/mL). The DP-CSVs of DB (1.0×10^{-5} mol L⁻¹) showed maximum peak current response at 0.1% (v/v) Nafion composition at -1.35 V *versus* Ag/AgCl electrode (Figure 4F). At this composition of Nafion, stable, reproducible current response and well-defined cathodic peak was attained. At Nafion composition in ethanol, less than 0.1% (v/v), unstable, ill-defined cathodic peak current and non-symmetric peak at -1.35 V was noticed at 0.1% (v/v). The current profile can be justified by the charge transfer resistance offered at higher contents (>0.1%) of Nafion due to formation of an progressively thick film that may , which may delay diffusion of DB diffusion as reported.³³ Moreover, the incapability of the grafted film of c-MWCNTs onto the surface of GCE may also account for the observed trend. At Nafion compositions greater than 0.1% (v/v), the peak current was significantly decreased (Fig. 4F). The formation of overly thick film of the nanocomposite onto the GCE is most likely minimizes reaching of the DB drug to the electrode surface, and consequently inhibiting the electrochemical reduction process of DB. Thus, in the following experiments, Nafion-ethanol (0.1% v/v)/c-MWCNTs (0.06 mg mL⁻¹) nanocomposite was selected for fabrication of the GCE.

The impact of volume of the nanocomposites of c-MWCNT (0.06 mg mL⁻¹)/Nafion-ethanol (0.1% v/v) is of great importance for DB determination by the established sensor. Thus, the impact of volume (2.0-14.0 μ L) of the nanocomposites of c-MWCNT (0.06 mg mL⁻¹

¹)/Nafion-ethanol (0.1% v/v) was also investigated. The plot of the DP-cathodic peak current at -1.35 V *versus* the volume of the nanocomposite is shown in Figure 4G. The cathodic peak current increased on increasing the volume of the nanocomposites up to 10 μ L of suspension (see Figure 4G). At volume higher than 10 μ L, the current response gradually decreased because of the increased thicknesses of the nanocomposite layer attached to GCE.⁴⁹ Moreover, nano composite suspension lowers than 10 μ L, the adsorbed DB species significantly decreased resulting in low cathodic current. The decrease of c-MWCNTs attached to the electrode surface limits the electron transport of DB, and consequently diminishes the current signal.^{48,49} Hence, 10 μ L of Nafion/c-MWCNT nanocomposite suspension was adopted for GCE modification.

3.4 Analytical performance

DP-CSV was adopted to assess the cathodic peak current response of different DB concentrations at the Nafion/c-MWCNTs under the optimum analytical parameters. The plots of the *cathodic peak currents* ($i_{p,c}$) of DP-CSVs recorded at -0.85 and -1.35 V at pH 7.0 varied linearly over a wide range (9.0×10^{-9} ($2.7 \mu\text{g L}^{-1}$) - 9.0×10^{-6} ($2.7 \times 10^3 \mu\text{g L}^{-1}$) mol L^{-1}) and 2.9×10^{-6} ($8.7 \times 10^2 \mu\text{g L}^{-1}$) - 8.0×10^{-5} ($2.4 \times 10^4 \mu\text{g L}^{-1}$) mol L^{-1}) of DB concentrations, respectively. Representative DP-CSVs of DB at various known concentrations at -1.35 V and the corresponding linear calibration plot (1~10 μ M) are illustrated in ESI. 7. At higher DB concentrations, the plot tended to level off because of the adsorption saturation of the modified surface with the target analyte.

The computed values of LOD ($3\sigma/b$) and LOQ ($10\sigma/b$)⁵⁰ for the proposed sensor at -0.85 V were found equal 2.7×10^{-9} ($8.1 \times 10^{-1} \mu\text{g L}^{-1}$) and 9.0×10^{-9} ($2.7 \mu\text{g L}^{-1}$) mol L^{-1} , respectively

where, σ is the standard deviation of the blank and b is the slope of the standard curve of the analyte. The figures of merits (LOD and LDR) of the proposed DB sensor were satisfactorily compared with various analytical methods (see Table 1). **It is evident that, the performance of the established sensor is better and simpler (drop-casting formation), low cost (without metal nanoparticles) and easier than most of the reported methods (Table 1).** The only disadvantage of the present method is the need for a regeneration step next to every measurement to remove the adsorbed DB at the Nafion/c-MWCNTs/GCE. On the other side, no electrochemical preconditioning is needed. The LOD of the settled procedure is below the maximum permissible limit (MPL) of anabolic steroids set by WHO and far below the limit assigned in drinking water.

Please insert Table 1

3.5 Selectivity and interference study

The influence of some inorganic and organic species at high concentration (100-fold excess of mass concentrations) on the electrochemical response of the cathodic peak current of DB (3.3×10^{-6} mol L⁻¹) at -1.35 V was studied at the optimized analytical parameters. The acceptance border was distinct as the concentration of the added external species producing a relative standard deviation (RSD) within $\pm 5\%$ of the magnitude of the cathodic peak current at -1.35 V vs. Ag/AgCl of the solution. The results revealed that, even at 100-fold excess of uric acid, ascorbic acid, citric acid, glucose, fructose, sucrose, methandienone steroid, starch at the potential of -0.932 V and 500-fold concentration of the ions Na⁺, K⁺, PO₄⁻³, NO₃⁻ and SO₄²⁻ did not interfere with DB analysis. The cathodic peak current of methandienone was recorded at -0.94 V and not overlapped with analyte peak at -1.35 V. A slight shift (± 0.05 V) of the cathodic peak potential was noticed without significant

interference. These results suggested the possible use of the developed sensor for analysis of DB drug in real samples e.g. biological fluids and environmental water samples.

3.6 Repeatability, validation and stability of the developed sensor

Repeatability: The repeatability of the Nafion/c-MWCNTs modified GCE was successfully evaluated by measuring various known concentrations of DB (5×10^{-6} and 5×10^{-5} mol L⁻¹) for 5 successive times with the same electrode under the same fabrication. The results revealed satisfying repeatability (RSD = 3.9-3.54 %), confirming the suitability of the electrode.

Intra- and inter day analysis: The stability of the proposed sensor towards analysis of DB was also tested by performing intraday consecutive measurements (n=5) of DB (5.0×10^{-5} mol L⁻¹). The average values of RSD were $\pm 4.23\%$, revealing the precision and suitability of the sensor. The repeatability of the sensor was also examined via inter day DP-CSV response at known DB concentrations (5.0×10^{-5} mol L⁻¹) over a period of five days. The results revealed good precision as noticed from the value the RSD ($\pm 3.9\%$) value.

Robustness: The established DP-CSV was tested for determination known DB concentration (5.0×10^{-6} mol L⁻¹) at minor changes in pH (7.4-8.0), accumulation time (55-65s) and sweep rate (60-80 mV) (Figure 4). The reliability (RSD = within $\pm 5.0\%$) of the cathodic peak current, consistency and precision of the developed DP-CSV were confirmed.

3.7 Analytical applications: analysis of DB in urine samples

Validation of the proposed sensor for analysis of DB in certified reference material (CRM) is of great importance. Due to the unavailability of CRM for DB, the planned sensor was successfully tested for analysis of DB in biological fluids (urine samples) using the method of standard addition. The measured DP cathodic peak current $i_{p,c}$ at - 1.35 V at the optimal

operational parameters, increased linearly at various additions of DB concentrations (0.0-10 $\mu\text{mol L}^{-1}$). The results are summarized in Table 2 and representative standard addition plots are shown in ESI. 8. The recoveries (%) of DB in the urine samples were found in the range ~97 to 102%. The experimental 't' value (1.67) (n=5) was less than the tabulated value (t=2.78) at 95% confidence⁵⁰ revealing precision and analytical utility of the established sensor for analysis of DB.

Please insert Table 2

4. Conclusions, advantages, limitations and future perspectives

The proposed DP-CSV approach has revealed substantial enhancement in the sensitivity, stability, and reproducibility of Nafion/c-MWCNTs modified GCE towards electrochemical activity of DB. The current study was successfully applied for analysis of DB in urine samples. Further, the LOD of the settled procedure was very low compared to other reported methods and it is remarkably lower than the MPL of anabolic steroids set by WHO in biological fluids and drinking water. The most common impurities in drinking water samples were negligible and did not hinder with DB detection by the proposed sensor. The main drawback of the current study is the need for a regeneration step next to every individual measurement to remove the adsorbed DB at the Nafion/c-MWCNTs/GCE. However, the method has distinctive advantages e.g. precise, short analytical time, sensitive, economical, reproducible and miniaturized sample preparation for DB analysis in biological samples of human origin. The present approach was favorably validated with the reported standard HPLC methods. The present sensor provides an excellent alternative method than the expensive chromatographic methods for trace analysis of DB in real samples. The existing approach can be promoted to sub-ppb levels of DB in biological fluids and wastewater

samples via online enrichment through nano-materials modified solid phase packed microextraction (SPME) ²¹ and/or dispersive liquid-liquid microextraction (DLLME).⁵¹ The present study also provides the basis for expansion of real-time handy doping detectors in near future. Experimental design will be recommended of follow-up manuscripts and it is much-admired in the future analytical strategies, because the one issue at a time **has** drawbacks and the interactive outcome of the various analytical parameters variations might improve the performance of the work.

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Compliance with ethical standards: The authors declare that they have no competing interests.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Figures Captions

Figure 1. SEM images of bare GCE (A), Nafion/GCE (B), Nafion/c-MWNTs modified GCE (C) and AFM (D) at magnification 100,000× at accelerating voltage 10 kV, and scale size 10 μm.

Figure 2. DP-CSVs of DB ($9.9 \times 10^{-5} \text{ mol L}^{-1}$) in phosphate buffer (pH 7.4) at bare GCE (1), and Nafion/c-MW-CNTs/GCE (2).

Figure 3. CVs of DB ($1.0 \times 10^{-4} \text{ mol L}^{-1}$) at pH 7.4 at 20 (a), 40 (b), 60 (c) and 80 (d) mVs^{-1} scan rate at Nafion/c-MWCNT/GCE. Inset: Plot of the cathodic peak current *versus* scan rate, mVs^{-1} .

Figure 4. Plot of the cathodic peak current of DB ($5 \times 10^{-5} \text{ mol L}^{-1}$) *versus* accumulation time (A), deposition potential (B), scan rate (C), pulse amplitude (D), mass composition of c-MW-CNTs (E), Nafion concentration (F) and suspension volume of Nafion/c-MW-CNTs (E). Conditions: pH: 7.4, Sweep rate = 60 mVs^{-1} and 0.05 V pulse amplitude.

Fig. 1

Figures Captions

Fig. 2

Fig. 3

Fig. 4

Table 1: Comparative figures of merits for determination of DB steroid by the established probe and some published methods ^a

Method	LDR (ng/mL)	LOD (ng/mL)	Reference
GC-MS-MS	40 – 4000	25	¹²
PF-MEKC	100 – 5000	59	¹³
LC-TOF	1 – 150	10	¹⁴
GC-MS-MS	-	10	¹⁵
SPME-LC-MS	0.05 – 100	3	²¹
UHPLC–MS/MS	5 – 1000	7.5	²²
MEKC	60 – 700	200	²⁷
DPV	2.0 – 60 ^a	0.43 ^b	²⁸
DP-CSV	3 – 2704	1.0	Present work

^a. DPV = Differential pulse voltammetry; MEKC =micellar electrokinetic chromatography, PF-MEKC; partial filling micellar electrokinetic capillary chromatography, LC-TOF; liquid chromatography- time of flight, GC-MS-MS; Gas Chromatography–Tandem Mass Spectrometry, SPME-LC-MS ;solid-phase microextraction - liquid chromatography-mass spectrometry. UHPLC–MS/MS; Ultra-high performance liquid chromatography–tandem mass spectrometry and DPV =differential pulse voltammetry

^b. Values are given in $\mu\text{mol/L}$.

Table 2: Determination of DB steroid in various urine samples by the proposed DP-CSV method ^a

Urine sample	Added ($\times 10^{-6}$ mol L ⁻¹)	Found ($\times 10^{-6}$ mol L ⁻¹)	Recovery (%)	t_{exp} ^b
I	1.99	1.99 $\pm$ 0.001	100 $\pm$ 1.4	0.05
	2.99	3.04 $\pm$ 0.002	102. $\pm$ 1.7	2.31
	3.98	4.06 $\pm$ 0.005	102.2 $\pm$ 3.0	0.95
	4.97	5.09 $\pm$ 0.005	102.4 $\pm$ 2.2	2.4
	5.96	5.78 $\pm$ 0.007	97.1 $\pm$ 2.7	2.5
II	2.99	2.94 $\pm$ 0.003	98.5 $\pm$ 4.5	0.72
	3.98	4.09 $\pm$ 0.004	102.8 $\pm$ 4.6	0.88
	4.97	5.02 $\pm$ 0.006	101.2 $\pm$ 5.0	0.41
	5.96	5.94 $\pm$ 0.0018	99.7 $\pm$ 1.4	0.39
	6.95	6.89 $\pm$ 0.004	99.26 $\pm$ 0.5	0.54

^a. Average recovery (% m/v) $\pm$ standard deviation (n=5).

^b. $t_{\text{critical}} = 2.78$ (P=0.05).