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Application of Carbon Nanotube-Graphite Mixture for the Determination of Diclofenac Sodium in Pharmaceutical and Biological Samples

Abdolmajid Bayandori Moghaddam[1], Ali Mohammadi [2,3]* and Mozhgan Fathabadi [2,4]

[1]Department of Engineering Science, College of Engineering, University of Tehran, P.O. Box 11155-4563, Tehran, Iran
[2]Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[3]Nanotechnology Research Centre, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, P.O. Box 14155-6451, Iran
[4]Pharmaceutical Quality Assurance Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

Abstract

In this study, the main purpose is to fabricate a sensitive and selective electrochemical sensor through the multi-walled carbon nanotube-graphite/Ag electrode (MWCNTs-G/Ag). The application of this sensor was developed for the determination of diclofenac sodium in pharmaceutical dosage form, urine and human plasma. MWCNTs-graphite mixture improved the electroactive surface area due to its porous structure and a remarkable increase in the peak currents was observed. It demonstrated a catalytic effect and speeded up the rate of redox process. Application of MWCNTs-G/Ag resulted in a sensitivity augmentation. It is found that a maximum current response for the sensor in the Britton-Robinson buffer solution can be obtained in pH 3. The prepared sensor showed good standard calibration curves during 3 consecutive days over the concentration range of 45-2000 ng/mL and RSD values ranging from 1.95–7.11%. The limit of quantitation and detection limit were 45 and 15 ng/mL, respectively.

Keywords: Multi-walled carbon nanotubes; Electro-oxidation; Diclofenac sodium; Biological fluids

Introduction

Carbon based electrode has been commonly used in electrochemical systems. In many aspects, it is an ideal material as electrode in the electrochemical experiments due to its attractive properties, including the good corrosion resistance, high electrical conductivity, low cost and a broad anodic potential window in aqueous media [1,2]. According to the degree of graphitization, it is morphologically diverse, existing in a various forms from carbon black to glassy carbon, carbon fibers and pyrolytic graphite [3]. Another interesting form of carbon, are carbon nanotubes (CNTs). They are allotropes of carbon with a cylindrical nanostructure. Allotropes of carbon, with their unique and fascinating one-dimensional nanostructure, are presently under examination as new tools for various applications [4-7]. The length-to-diameter ratio of CNTs is considerably larger than other materials. CNTs are assigned as two typical classes: single-walled (SWCNTs), and multi-walled carbon nanotubes (MWCNTs) [8]. SWCNTs consist of a single graphitic sheet rolled up into a cylindrical form, while MWCNTs are composed of concentric graphite tubules. They show extraordinary strength and unique electrical properties and can be considered as attractive candidates in diverse nanotechnological applications. Furthermore, CNTs illustrate wide potential window, chemical inertness, low cost and suitability for various sensing and detection. The subtle electronic properties suggest that CNTs have the ability to promote electron-transfer reactions, when used as an electrode in chemical reactions [9,10]. Carbon nanotubes have been widely used in electrochemical studies, where carbon nanotube modified electrodes employed for sensing applications. Recent studies showed that modified electrodes by carbon nanotubes and other nanostructures could impart electrocatalytic activity to the electrochemical studies [11-13].

Diclofenac, 2-(2,6-dichloranilino) phenylacetic acid, is a non-steroidal anti-inflammatory drug. It is used mainly as a sodium salt for the relief or pain and inflammation in various conditions: musculoskeletal and joint disorders such as rheumatoid arthritis, osteoarthritis, spondylarthritis, ankylosing spondylitis [14].

An additional indication is the treatment of acute migraines. Diclofenac is used commonly to treat mild to moderate post-operative or post-traumatic pain, particularly when inflammation is also present, and is effective against menstrual pain and endometriosis [15]. It has been determined by a variety of analytical techniques, such as spectrophotometry [16], reflectometry [17], thin-layer chromatography [18,19], gas chromatography [20,21], high-performance liquid chromatography-mass spectrometry [22], high performance liquid chromatography [23], capillary zone electrophoresis [24], polarographic [25], and potentiometric analysis [26]. Recently, some sensors reported for the diclofenac analysis [27,28]. In addition, Blanco-Lopez et al was developed voltammetric sensors for the determination of diclofenac, based on the molecular recognition of the analyte by molecularly imprinted polymers [29,30]. Most of these methods require either sophisticated instruments or expensive reagents or involve several manipulation and derivatization steps. To the best of our knowledge, there is very little voltammetric methods reported in the literature for determination of diclofenac sodium [31]. The voltammetric methods are very interesting and show to have several advantages for the sensitive determination of a range of drug compounds [32-35]. In electrochemical techniques, sample preparation usually consists in dissolving the active compound from the pharmaceutical dosage forms in a suitable solvent and performing a direct analysis on an aliquot of this solution. The specificity and selectivity of the voltammetric

***Corresponding author:** Ali Mohammadi, Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran, E-mail: alimohammadi@tums.ac.ir

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techniques are usually excellent because the analyte can be readily identified by its voltammetric peak potential.

In this work, MWNTs-graphite/Ag electrode was prepared to achieve a high electroactive surface area for development of an electrochemical sensor for the determination of diclofenac sodium. This electrode has the advantages of easy preparation, rapid and simple operation, reproducibility and very low interference and high accuracy in tablets and biological samples. Some experimental parameters including, pH, scan rate, and electrochemical technique were scrutinized to find the best function of the sensor. The proposed method was successfully applied to determine diclofenac sodium in pharmaceutical formulation and biological fluids.

Experimental Section

Chemicals and materials

All chemicals used in this work were of analytical-reagent-grade chemicals from Merck Co. (www.merck.com). The multi-walled carbon nanotubes (MWCNTs) were purchased from Neutrino Co. (www.neunano.com, Iran). The MWCNTs had an outer wall diameter distribution of <10 nm, a length of between 5-15μm and amorphous carbon <3%. Distilled water was used to prepare all solution and in all experiments. The 0.04 M Britton-Robinson (B-R) buffer solutions of pH 3-8 ranges were freshly prepared. Buffer solutions (pH 3-7) were prepared by mixing corresponding amounts of 0.04 mol/L H_3BO_3, 0.04 mol/L CH_3COOH, 0.04 mol/L H_3PO_4 and 0.2 mol/L NaOH. Diclofenac sodium was obtained from Iranian Quality Control Lab Ministry of Health and Medical Education (www.behdasht.gov.ir). Fresh frozen plasma was obtained from Iranian Blood Research and Fractionation Holding Company (http://ibrf.ir/EN/Concern.asp). Drug free human urine was obtained from healthy volunteers (25-30 years).

Apparatus

All the electrochemical measurements were performed by the μ-AUTOLAB TYP III (www.metrohm-autolab.com). A three-electrode cell was also used, the working electrodes were glassy-carbon (GC) and MWCNTs-G/Ag electrode. A Pt wire and an Ag/AgCl/KCl (sat.) (both from Azar Electrode Co., Iran) were used as the counter and reference electrodes, respectively. (KCl-saturated, 0.197 V versus a normal hydrogen electrode (NHE)). All experiments were performed at 25 ± 1°C. The sonication was performed by using an ultrasonic bath system TECNO-GAZ, Tecna 6 (50–60 Hz, 230 ± 10% V, 0.138 KW). Furthermore a Philips model X-30 scanning electron microscope was used to capture images.

Fabrication of carbon nanotube-graphite mixture

Before modification, the surface of Ag electrode was prepared by polishing on a polishing cloth with aqueous alumina slurries. The remaining particles on the surface were removed by ultrasonic treatment in ethanol for a few minutes. Finally it was rinsed with doubly distilled water. This work was carried out for every renewal paste on Ag electrode. For making paste the MWCNT:graphite-based electrode was prepared from a mixture of MWCNTs and graphite powder with different weight ratio. After weighing carbon and Graphite, they mixed together truly for 10 min and then the mixture was added to paraffin oil and again the mixture was mixed for 15 min. A thin layer of the paste was packed into the end of an Ag electrode. Table 1 shows the amount of each component for making different pastes.

Preparation of standard solutions

A suitable amount of working standard powder of diclofenac sodium was dissolved in 25mL distilled water to form a stock solution at a concentration of 5mM. Additional dilute solutions with different concentrations in different pHs were prepared daily by accurate dilution by B-R buffer. The stock solution was protected from light using aluminium foil and stored at 4˚C for three days. Plasma and urine standard solutions (200 ng/mL, 1000 ng/mL and 2000 ng/mL) were prepared by spiking of aqueous stock solution of diclofenac to drug free plasma and urine samples.

Extraction procedure

For the determination of diclofenac sodium in biological fluid 0.5mL of 0.5 M HCl was added to 0.5 mL plasma/urine standard solution and vortexed for 2 min. This mixture was then blended with 5 mL ethyl acetate, vortexed for 3 min. and centrifuged at 4500 rpm for 5 min. After removal of the organic phase the extraction was repeated on the residual aqueous phase. The ethyl acetate phase were pooled and dried at 60 ºC under a gentle stream of nitrogen. After drying, samples were diluted with 20 mL of B-R buffer in pH 3 and transferred to electrochemical cell for analysis.

Tablet assay procedure

For the analysis of diclofenac tablets, 20 tablets were weighed and powdered well in a pestle. An appropriate, accurately weighted amount of the powder equivalent to the weight of one tablet was dissolved in doubly distilled water. Finally, aliquots of this solution were diluted with B-R buffer at PH 3 to obtain a concentration of 1000 ng/mL.

Validation of the method

Validation of this procedure for the quantitative assay of the drug was examined via evaluation of the linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy and selectivity. Square wave voltammograms (SWVs) of diclofenac solutions with concentrations range (45-2000 ng/mL) at pH 3 at MWCNTs-graphite/Ag electrode (1:1) in B-R buffer were recorded.

paraffin oil (μL)	MWCNTs (mg)	graphite powder (mg)	electrode
3.6	-	12	graphite
2.7	3	6	MWCNTs-G (1:2)
2.7	6	3	MWCNTs-G (2:1)
3.6	6	6	MWCNTs:G (1:1)

Table 1: The components of electrodes.

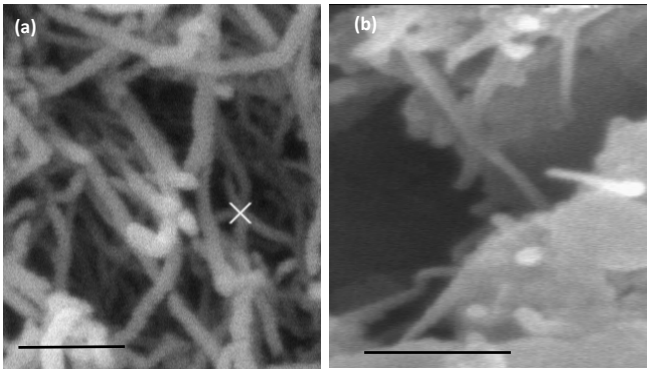

Figure 1: SEM images of **a** MWCNTs and **b** MWCNTs-G mixture (1:1), scale bars are 500 nm.

Results and Discussion

Characterization of MWCNTs and MWCNTs: graphite mixture

The microscopic structure of MWCNTs and MVCNTs-graphite mixture were characterized using SEM images. (Figure 1a) shows SEM image of MWCNTs while (Figure 1b) shows SEM image of MWCNTs-graphite mixture. The presence of paraffin oil in MWCNTs - graphite mixture could bridge the isolated carbon materials and MWCNTs could be clearly observed in (Figure 1b).

Voltammetric studies of the drug on different electrodes

In this study, voltammetric methods such as cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were carried out at pH range 3-7 in diclofenac solutions 15000 ng/mL at both GC and MWCNTs-graphite/Ag electrodes with different ratios of MWCNTs-G/Ag electrode in B-R buffer. The results show that the best response is obtained on MWCNTs-G/Ag electrode with ratio of (1:1). So this paste was chosen for analyzing of diclofenac in this study. By overlaying CVs and DPVs in different pH at different electrodes (the results are not showed) there was found that the best pH was 3.

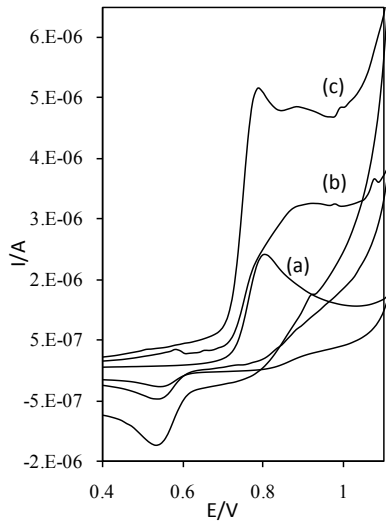

Figure 2: Comparative CVs of diclofenac sodium (15000 ng/mL) in B-R buffer (pH 3) at different electrodes; **a** glassy carbon, **b** graphite, **c** MWCNTs-G/Ag electrode (1:1).

The peak potential (Ep) shifts to more negative values with the pH increase. The peak current had higher values in acid solutions, and decreased with increasing pH. Taking into account that from an analytical point of view high peak currents together with low oxidation potentials are desirable, this should be a good pH region for analytical determinations. For investing drug's stability in acidic media, t-test [36] was done in 3 days continuously in concentrations of 500 ng/mL and 2000 ng/mL in pH 3. The values of $t_{calculated}$ were then compared to a $t_{tabulated}$ with 4 degrees of freedom at the 95% confidence level (t =2.776). The calculated t-values (2.66 and 0.93 for concentration of 500 ng/mL and 2000 ng/mL, respectively) were smaller than the theoretical ones. These results indicate that drug is stable in pH 3.

The enhanced voltammetric behaviour of diclofenac sodium at MWCNTs-G/Ag electrode

The electrochemical oxidation of 15000 ng/mL diclofenac sodium at the MWCNTs-G/Ag electrode in B-R buffer solution (pH 3.0) was examined by cyclic voltammetry (CV). The cyclic voltammograms are illustrated in (Figure 2). A well-defined oxidation peak is observed at about 0.8 V. In order to illuminate the enhancement properties of MWCNTs-G/Ag electrode for the oxidation of diclofenac sodium, CVs of 15000 ng/mL diclofenac sodium in B-R buffer pH 3 are tested at different working electrodes including Graphite (curve a), bare GCE (curve b), MWCNTs-G/Ag electrode (curve c) in (Figure 2a-c). The peak current of diclofenac sodium greatly increases at MWCNTs-G/Ag electrode. It is believed that MWCNTs show highly effective enhancement to diclofenac sodium oxidation because of its large specific surface area and subtle electrical properties, which provides enough effective reaction sites to increase the electron exchange rate.

Effect of pH on the peak potentials and peak currents

The peak potential and the peak current are closely depending on the pH of solution. The cyclic voltammograms of 15000 ng/mL diclofenac in buffer solution in the range of pH from 3 to 7 at MWCNTs-G/Ag electrode are obtained (Figure 3a). The obtained voltammograms demonstrate that in pH 3 we have the sharpest response rather than other pH values. The (Figure 3b) shows a linear relationship between pH values and anodic peak potentials (Epa), a linear shift of the

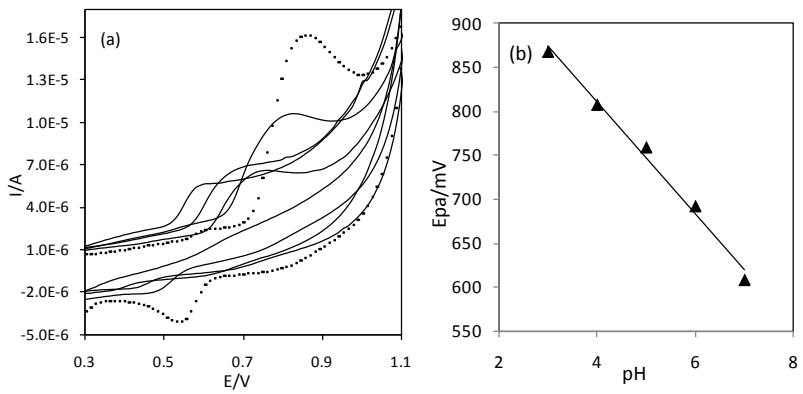

Figure 3: a CVs at MWCNTs-G/Ag electrode (1:1) in B-R buffer at various pH (right to left); 3, 4, 5, 6 and 7, scan rate: 80 mV/s. **b** Relationship between pH and anodic peak potential.

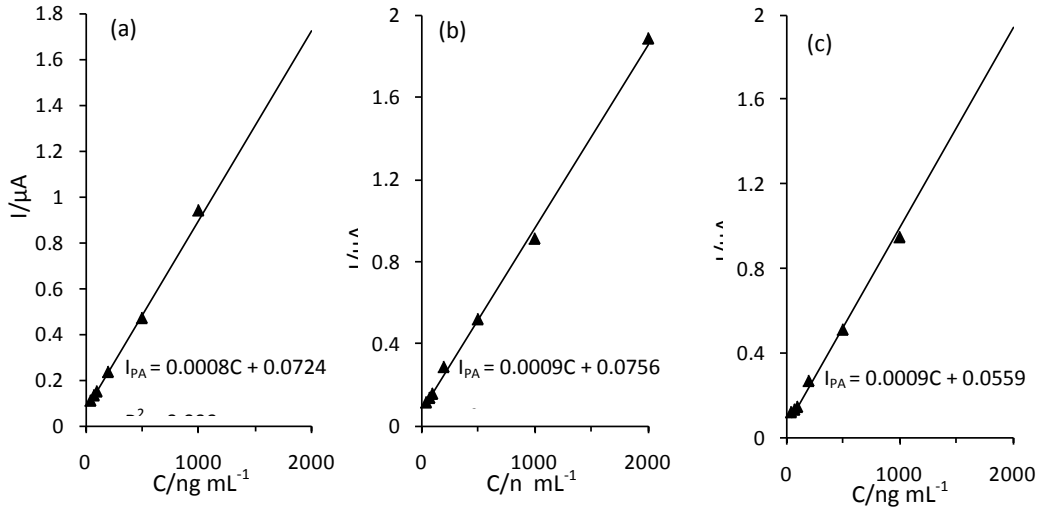

Figure 4: Plot of peak current vs. concentration of diclofenac sodium in B-R buffer (pH 3) for various concentrations; 45, 75, 100, 200, 500, 1000 and 2000ng/mL.

Calibration curve (µM)	LOD (µM)	Electrode type	Electrochemical technique	Reference
-	-	non-imprinted polymeric modified electrode	cyclic voltammetry	30
6.0 - 50.0	4.3	bismuth film electrodes	amperometry	39
0.17 - 2.5, 2.5 - 75	0.08	MWCNTs-dihexadecyl hydrogen phosphate film	cyclic voltammetry chronocoulometry	40
9.90 - 500	3.8	carbon fiber microdisk array electrode	capillary zone electrophoresis	41
196 - 525	27.9	Ni–curcumin complex modified GC electrode	cyclic voltammetry, chronoamperometry	42
9.90 - 500	2.5	carbon fiber array electrode	capillary zone electrophoresis	24
196 - 2650	31.7	nickel hydroxide-modified nickel electrode	cyclic voltammetry, chronoamperometry	43
0.14 - 6.29 (45-2000 ng/mL)	0.047 (15 ng/mL)	MWCNTs-G/Ag electrode	square wave voltammetry	[this work]

Table 2: Different reported electrodes for determination of diclofenac.

peak potential towards more negative values was observed as the pH increased from 3 to 7. Epa decreased by about 63.54 mV per pH, with equation of Epa = -63.54 pH + 1064.6 (R^2 = 0.99). The slope of 63.54 mV per pH was close to the theoretic value 59 mV per pH, suggesting one electron and one proton transferred in the oxidation of diclofenac sodium. The oxidative reaction of diclofenac sodium is a one electron, one-proton transfer process resulting in the radical species [37,38].

Calibration curves

A typical calibration curve for diclofenac was demonstrated in (Figure 4a-c). The relationship between diclofenac sodium concentration and the oxidation peak current can be described with the following linear regression equations in the range of concentration from 45 ng/mL to 2000 ng/mL at 3 consecutive days:

Ipa = 0.0008C + 0.0724 (R^2 = 0.999)

Ipa = 0.0009C + 0.0756 (R^2 = 0.998)

Ipa = 0.0009C + 0.0559 (R^2 = 0.998)

According to the obtained results, the best regression equation for the calibration curve was found to be Ipa = 0.0008C + 0.0724 (R^2 = 0.999). The relative standard deviation (%RSD) values ranging from 1.95–7.11% across the concentration range studied were obtained. Under the optimized experiment conditions described above, LOQ and LOD of diclofenac sodium were obtained to be 45 ng/mL and 15 ng/mL, respectively.

Repeatability, stability and reproducibility of the modified electrode

Precision of the method was investigated with respect to both repeatability and reproducibility. The repeatability of the modified electrode was investigated by repetitive recording at a fixed diclofenac concentration of 15000 ng/mL. A decrease for the peak currents in CVs based on 7 replicates was 1.05% indicating good repeatability of the response of modified electrode with the %RSD value less than 2.54. The reproducibility was evaluated by measuring the oxidation current values for fresh solutions of each of the 45, 1000 and 2000 ng/mL standards over a period of 3 days. The mean concentrations were found to be 48.32, 978.02 and 2027.12 ng/mL with associated %R.S.D. values of 7.11, 1.95 and 3.2, respectively. Accuracy of the assay was determined by interpolation of replicate (n=3) peak areas of three accuracy standards (45 ng/mL, 1000 ng/mL and 2000 ng/mL) from a calibration curve prepared as previously described. In each case, the percent relevant error was calculated. The resultant concentrations were 47.28 ± 2.95 ng/mL (mean ± S.D.), 1044.07 ± 38.57 ng/mL and

1972.99 ± 28.65 ng/mL with percent relevant errors of 5.1, 4.41 and -1.35%, respectively.

Response characteristic

Some voltammetric procedures have been reported for the determination of diclofenac in pharmaceutical tablets and biological fluids. But in accordance to our knowledge, there is no report regarding the use of MWCNTs-graphite/Ag electrode for the determination of diclofenac. By this electrode, we could determine diclofenac with the LOD value of 15 ng/mL which is the lowest of LOD for the determination of this drug at different electrodes until now. In (Table 2), the response of the proposed method is compared with those obtained by reported methods. The results showed that Ag electrode modified with carbon nanotubes is suitable for sensitive and selective determination of diclofenac.

Determination of diclofenac in pharmaceutical samples

The applicability of the proposed voltammetric method for pharmaceutical dosage forms was examined by the analyzing the tablets. The result of the assay of diclofenac tablets yielded a recovery of 101.03% (R.S.D. =2.35%) of label claim for the tablets. In order to evaluate the accuracy of this method and to know whether the excipients in pharmaceutical dosage forms show any interference with the analysis, the proposed voltammetric method was checked by recovery experiments using the standard addition method. After addition of known quantities of analyte to the drug product, the mixture was analyzed by the proposed method. The recovery of diclofenac was calculated using the corresponding regression equations of previously plotted calibration curves. The results of recovery experiments are presented in (Table 3). The results indicate the absence of interference from commonly pharmaceutical excipients used in the selected formulations. Therefore, the method can be applied to the determination of diclofenac in pharmaceutical forms without any interference from inactive ingredients.

Sample	Amount labeled (mg)	Amount added (mg)	Amount found (mg)	Recovery (%)
A	25	-	25.31	101.24
B	25	5	30.61	102.03

Table 3: Determination and recovery of diclofenac sodium in commercial tablets.

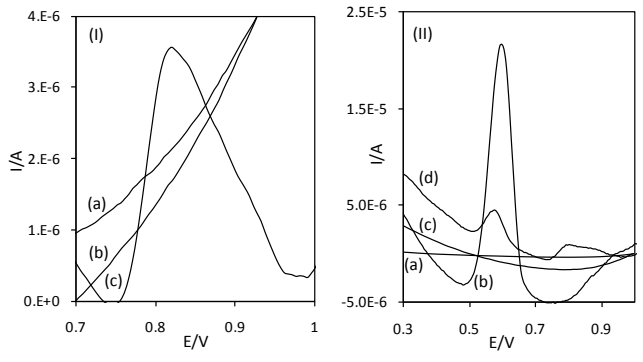

Figure 5: (**I**) SWVs of the **a** buffer, **b** extracted blank plasma **c** extracted plasma spiked sample (2000ng/mL). (**II**) SWVs in **a** buffer **b** urine blank previous extraction, **c** urine blank after extraction and **d** extracted urine spiked sample (2000 ng/mL). Frequency = 50 Hz, pulse amplitude = 30 mV and step potential = 5 mV.

Spiked diclofenac	200 ng/mL	1000 ng/mL	2000 ng/mL
Recovery percent (mean ± S.D.)	77.82 ± 4.88	75.09 ± 1.95	77.51 ± 2.15

Table 4: Recovery percent of diclofenac sodium electrochemical determination in plasma (n = 3) by MWCNTs-G/Ag electrode.

Spiked diclofenac	200 ng/mL	1000 ng/mL	2000 ng/mL
Recovery percent (mean ± S.D.)	87.34 ± 5.25	80.43 ± 4.35	70.67 ± 2.18

Table 5: Recovery percent of diclofenac sodium electrochemical determination in human urine (n = 3) by MWCNTs-G/Ag electrode.

Determination of diclofenac in human plasma

The prepared modified electrode was also applied to the analysis of the human plasma samples using SWV method. The drug-free plasma samples were spiked with different amounts of standard diclofenac and their SWV were recorded using the modified electrode. In our tests, no interference peak was detected in healthy plasma. In (Figure 5I) the buffer and blank plasma are shown. Three concentrations of diclofenac spiked plasma were tested (200, 1000 and 2000 ng/mL). The results of recovery evaluations have been shown in Table 4)

Determination of diclofenac in human urine

The practical analytical application of the proposed method was further established by estimation of diclofenac in human urine samples. The voltammograms of human urine sample without and with the standard solution of diclofenac sodium are shown in (Figure 5II). To determine the extraction efficiency, diclofenac sodium was spiked in urine at concentrations of 200, 1000 and 2000 ng/mL and extracted using the stated extraction procedure. The results obtained are listed in Table 5.

Conclusion

MWCNTs have large specific surface area and strong adsorptive properties providing more reaction sites. MWCNTs-G paste on the electrode surface is porous. Because of these characteristics the adsorption of diclofenac sodium to the electrode surface becomes easy. Correspondingly, the concentration of diclofenac sodium at the electrode surface was enlarged. So the oxidation peak current of diclofenac sodium was greatly enhanced at MWCNTs-G paste modified electrode. In this study, several ratio of carbon nanotube - graphite paste electrode were made and voltammetric studies of them were compared to GCE. The best paste was carbon nanotube - graphite with ratio of (1:1) that other studies focused on it. We had the lowest LOD (15 ng/mL) for diclofenac determination compare to other electrodes which have been reported until now. High sensitivity and improved detection limit of the MWCNTs-G/Ag electrode are promising for the determination of diclofenac content in biological fluids as well as pharmaceutical preparations.

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