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Original Article

Novel electrochemical quantification for nepafenac in raw drug, pharmaceutical eye drops and biological fluid

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ABSTRACT

This article pronounces differential pulse voltammetric technique for the determination of nepafenac in pharmaceutical dosage form and human serum. The proposed voltammetric technique was established on electro-oxidation of nepafenac at two electrodes, the first electrode is the carbon paste modified with 10% w/w of 1-n-butyl-3-methylpyridinium hexafluorophosphate ion crystal (10%BMH-CPE) and the second electrode is carbon paste modified with 10% w/w of each of multi walled carbon nanotubes and 1-n-butyl-3-methylpyridinium hexafluorophosphate (CMWNT-BMH-CPE).The method was performed in 0.04M B-R electrolyte with pH=6.0. The peak current concentration relationship was linearand the suggested methods were applied successfully to the pharmaceutical dosage form and spiked human serum with good repeatability and reproducibility.

Keywords: Nepafenac; Ionic liquid crystal; carbon multi walled nanotubes; biological fluid.

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INTRODUCTION

Nepafenac (NPF), 2-amino-3-benzoyl benzene acetamide, is non-steroidal anti-inflammatory drug (NSAID) prepared as an ophthalmic suspension dosage form. NPF is the pro-drug of amfenac, very effective nonselective cyclo-oxygenase COX-1 and COX-2 inhibitor. It is used to treat and prevent ocular pain and inflammation that can be present after cataract surgery by lowering the production of prostaglandins in the eye [1, 2].

The removal of diclofenac sodium ophthalmic solution as a viable pharmaceutical entity in September 1999 from the US market gained considerable interest in the general safety and effectiveness of topical ophthalmic NSAIDs for treatment of anterior segment inflammation. In late 1999 the use of topical ocular NSAIDs decreased in the US as result of incidents involving corneal melts and toxicity surrounding use of generic diclofenac. However, since removal of diclofenac sodium ophthalmic solution from the market place, ophthalmic NSAIDs have regained use as viable pharmacotherapeutic entities. Moreover, new ophthalmic NSAID products have recently been introduced for commercial use in the US including the novel chemical entity NPF [3].

The purpose of this report is to revisit the use of topical ophthalmic NSAIDs for the treatment of surgically induced anterior segment inflammation with focus on NPF. NPF is a prodrug deaminated to amfenac, a highly effective non-selective cyclo-oxygenase inhibitor. In the case of topical ophthalmic NSAIDs, practitioners should carefully weigh the cost-benefit of implementing "highly potent" new drug products because perturbations in pharmacodynamic response due to the inherent novelty in terms of chemical designs may outweigh the demonstrated replicative pharmacologic action of all topical ophthalmic NSAIDs [3].

NPF was determined in ophthalmic dosage form using several methods including UV spectrophotometry [4-6], HPLC [6-9]. Many of the reported methods require the use of sophisticated equipment and expensive reagents. Some are exhausting, requiring prolonged sample pretreatment, strict control of pH and long reaction times. Chemically modified electrodes (CMEs) have recently gained interest due to their prominent advantages such as providing noticeable peak current and lowering overpotential for redox systems. Modification of electrodes with several modifiers such as transition metal complexes [10], nanostructures [11], molecular sieves [12] and organic compounds [13] has been reported in recent years. The carbon paste electrode (CPE), which is made of graphite and organic liquids, has been widely applied in the electroanalytical field because its cheap, easily fabricated, highly sensitive and possesses renewable surface [14–16]. To improve the sensitivity, selectivity, detection limit and other parameters of CPE, chemically modified carbon paste electrodes have been used [17–19]. The operation mechanism of such depends on the properties of the modifier used to improve selectivity and sensitivity for the target drug [20].

Ion liquid crystals have importance recently as it improves determination of drugs in voltammetry. Carbon electrodes modified with ionic crystals introduce benefits over ordinary CPEs, such as marked conductivity and sensitivity and rapid electron transfer. Ionic liquid modified electrodes proved good electro catalytic activities and used in several applications as in electrochemistry [21]. Gold nanoparticles, with large surface area, better biocompatibility, improved conductivity and electro catalysis properties, have been used to improve the detection limits in electrochemical studies [22–26]. They are suitable for surface immobilization and can act as tiny conduction centers and facilitate electron transfer [15,16, 27–32].

The objective of this study is to determine nepafenac (NPF) in bulk powder, pharmaceutical dosage form and biological fluid using two electrodes, the first electrode is the carbon paste modified with 10% w/w of 1-n-butyl-3-methylpyridinium hexafluorophosphate ion crystal (BMH-CPE) and the second electrode is carbon paste modified with 10% w/w of each of multiwalled carbon nanotubes and 1-n-butyl-3-methylpyridinium hexafluorophosphate (CMWNT-BMH-CPE).

EXPERIMENTAL

Chemicals and reagents: Nepafenac (NPF) was supplied from Orchidia company, potency was certified to be 101.7 %. Nevaxal® eyedrops labeled to contain 1 mg/5 mL NPF from Orchidia company (Cairo, Egypt) were purchased from local market. A stock solution of 1mg/mL NPF was freshly prepared by dissolving the weighed amount 50 mg in 50 ml bi-distilled water and stored in refrigerator 4^{o}C between measurements. Carbon multiwalled nanotube (CMWNT), 3-20 nm OD, 1-3 nm ID, 0.1-10 micron long 95% powder and 1-n-butyl-3-methylpyridinium hexafluorophosphate ion crystal (BMH), were all purchased from Alfa Aesar, Germany. Britton- Robinson (B-R buffer) of concentration 0.04 M was prepared by mixing phosphoric acid, acetic acid and boric acid [33]with appropriate amount of 0.1 M NaOH to obtain the

desired pH range (2-9). All solutions were prepared from analytical grade chemicals and deionized water. All materials and reagents were used as received without further purification.

INSTRUMENTAL AND EXPERIMENTAL SET UP

Voltammetry Measurement: Voltammetric measurements were obtained using the electrochemical analyzer computrace system with 797VA, computrace software (1.0) from Metrohm, Switzerland. The three electrodes system consisted of working electrode, Ag/AgCl (3M KCl) electrode as the reference electrode, and a platinum wire as the auxiliary electrode. Glassy carbon electrode, mini glassy carbon disk electrode of the active zone: 2.8 mm, for ELCD 641/656.A JENWAY 3510 pH meter (England) with glass combination electrode was used for pH measurements. The pH was calibrated using standard buffer pH 4.0, pH 7.0and pH 10.0. A Mettler balance (Toledo-AB104) was used for weighing the solid materials, U.S.A. A micropipette (Eppendorf- multipette plus) was used throughout the present experimental work, German. Ultrasonic Cleaner, United Jeveiry Tool Supplies, model UTA-60, 6L capacity, Italy. Deionized water used throughout the present study was supplied from a burette still plus deionized connected to a Hamilton-Aquametric deionized water system, U.K. All experiments were carried at an ambient temperature of 25±1°C.

PREPARATION OF THE ELECTRODES

Preparation of carbon paste electrode modified with 1-n-butyl-3-methylpyridinium hexa fluorophosphate (BMH) ion crystals (BMH-CPE): Carbon paste electrodes modified with different ratios (5, 10, 15, 20 and 25) % of BMH ion crystals were prepared by hand mixing suitable amounts of BMH ion crystals to get the desired percentage and complete to 225 mg with graphite powder and pasted with 14.5 mg paraffin oil in an agate mortar to get homogeneous carbon paste. The carbon paste was packed into the hole of the electrode body and smoothed on a filter paper until a shiny appearance was achieved.

Preparation of carbon paste electrode modified with multiwalled carbon nanotubes (CMWNT) and 1-n-butyl-3-methylpyridinium hexa fluorophosphate ion crystal (BMH) ion crystals (CMWNT- BMH - CPE): Carbon paste electrode modified with10% CMWNT and10% BMH ion crystals was prepared by hand mixing a ratio of 22.5 mg w/w CMWNT and 22.5 mg w/w BMH ion crystals then completed to 225 mg with graphite powder and pasted with 14.5 mg paraffin oil in an agate mortar to get homogeneous carbon paste. The carbon paste was packed into the hole of the electrode body and smoothed on a filter paper until a shiny appearance was achieved.

RECOMMENDED EXPERIMENTAL PROCEDURES

Assay of pure form: In the electrochemical measurements, Voltammetric analyses were performed in 15 ml B-R buffer pH=6.0for all prepared electrodes. Appropriate aliquots of the drug solution of NPF were introduced into the electrolytic cell while 8 mm of CPE was immersed into the supporting electrolyte. Calibration curves of NPF using differential pulse voltammetric technique (DPV) was constructed by plotting the peak current (IμA) against drug concentration (μg/mL). The anodic peakthat appeared for NPFwas around 900 mV for (CMWNT-BMH-CPE)and around 930 mVfor (BMH-CPE). The peaks were sharp symmetrical and smooth without noise with the calibration experiment.

Analysis of Pharmaceutical dosage form: A portion of 1.5 mL suspension of Nevaxal® eyedrop were carefully transferred in a 5 mL volumetric flask and diluted to the mark with methanol then filtered to prepare a stock concentration of 60 μg/mL. The amount of NPF was calculated using the linear regression equation obtained from the calibration curve of pure NPF [34].

Application to spiked human serum samples: Drug-free human blood samples got from healthy volunteers (after having obtained their written consent), was centrifuged (4000 rpm) for 15 min at room temperature and separated serum samples were stored frozen until assay. After thawing, an aliquot appropriate volume of sample was fortified with NPF dissolved in bi-distilled water to required concentration and preserved with 0.5 ml of acetonitrile as serum protein precipitating agent, then the volume completed to 2 ml with serum sample. The tubes were vortexed for 50 sec. and then centrifuged 5 min. at 4000 rpm for getting rid of protein residues. The supernatant was taken carefully. Appropriate volume of supernatant liquor was transferred in the voltammetric cell containing supporting electrolyte. Voltammograms were recorded as in pure NPF. Different amounts of acetonitrile were tried. The best results were obtained with 0.5 ml acetonitrile. The concentration of NPF was varied in human serum samples. Quantifications were performed by means of calibration curve method from the related calibration equation.

Standard addition technique: Standard addition technique was applied by adding different additions of standard solution to a fixed concentration of

drug dosage solution. The amount of NPF was calculated using the linear regression equation obtained from the calibration curve of pure NPF[34].

RESULTS AND DISCUSSION

Electrode surface morphology study: The surface morphology of modified sensors greatly affected the efficiency and the catalytic performance headed for NPF oxidation. Fig. (2A-C) shows the SEM of (CPE), (10%BMH-CPE) and (CMWNT- BMH-CPE), respectively. The SEM of (CPE) electrode displayed discrete asymmetrical graphite peel (Fig 2A). The presence of BMH achieved a blurred character with superior surface area, modest viscosity and marked high conductivity (Fig 2B). The presence of the exceedingly conductive ionic liquids crystal (BMH) between the graphite flakes affected greatly the conductivity of the paste and moreover, achieved more well-ordered films because of its distinctive molecular orientation so BMH plays main dual roles as a binder and a link or ions transferor among the graphite fragments improving the conductivity of the film. Moreover, modification with CMWNT formed smooth superficial nanostructures which markedly increased the conductivity (Fig 2C).

Electrochemical behavior of NPF: CV technique was employed to study the reversibility of the oxidation process of NPF. The cyclic voltammograms showed anodic peak with no peak on the reverse scan, suggesting the irreversible nature of the electrode reaction as depicted by the cyclic voltammograms. To determine which electrode has maximum sensitivity for NPF, DPV response was studied for the electrodes, (different ratios of BMH-CPE) and (CMWNT-BMH-CPE). The voltammograms revealed the highest response is for (CMWNT-BMH-CPE) followed by (10% BMH-CPE) as shown in Fig.3. The increased peak current indicated that CMWNT contributed to the drug electrocatalysis by increasing the surface area. Also, BMH helps the direct electron transfer between the drug and the bulk electrode surface. Anionic BMH has an electrostatic attraction with the cationic drug, that enhances the diffusion of drug through the electrode surface. Also, there is interaction between the positively charged drug and anionic BMH which enhances hydrogen bond formation between drug and BMH. Accordingly, both electrodes (CMWNT- BMH- CPE) and (10% BMH-CPE) were selected for subsequent determinations and method validation for nepafenac.

Mechanism: In the proposed method, the electro-oxidation of NPF involves one electron and one proton transfer process, the plausible mechanism is as shown in Fig 4. Here the amino group (-NH2) is attached to the carbon atom (C-2) of the benzene ring. During electrolysis when the first proton is removed, nitrogen losses one negative charge and an anionic species is formed. To stabilize the anionic form, hydrogen atom attached to the carbon (C-2) of the cyclohexane undergoes further electro oxidation and stable product 7-oxo paclitaxel is formed.

Effect of Operational Parameters

Effect of solution pH: The effect of electrolyte pH on the oxidation of NPF at (CMWNT-BMH-CPE) was studied by the differential pulse voltammograms technique using B–R buffer within the pH range of (2–9) shown in Fig 5 (A-C). The anodic peak potentials lifted negatively with the rise in the electrolyte pH, indicating that the oxidation of NPF is a pH-reliant reaction and displaying that protons have occupied part in the electrode reaction routes. The correlation between the anodic peak potential and the solution pH value with the pH range of (2–9) could be fitted into the linear regression equation E (V) = 0.944-0.011 pH, with a correlation coefficient r = 0.9995. NPF anodic current responses gave highest value at pH 6 and at low pH values the current responses were higher than those at high pH values; this is due to the pKa value of NPF which is 9.08 therefore, NPF can be attracted by the negative charges of the electrode. The highest oxidation peak current was obtained at pH 6.0. Thus pH 6.0 was employed for the determination of NPF to achieve higher sensitivity.

Effect of scan rate: Effect of different scan rates on the current response of 20 µg/mL NPF at (CMWNT-BMH- CPE) in 0.04M B–R buffer (pH 6.0) was investigated (Fig 6). A plot of current peak height (I µA) versus ofscan rate (ν) resulted in straight linear relation for scan rate of 20 to 300 mV s-1 with correlation coefficient r = 0.9992(Fig 6). This indicated that the charge transfer was partially under adsorption control. The redox peakcurrents increased with the linear regression as: I (A) = 6.19 V + 0.91 (r = 0.9992).

Mode of Scan: To develop a voltammetric method for determining the drug NPF, we selected the DPV since the peaks are sharper and well defined with improvedresolutionat lower concentrations of NPF than those obtained by CV. Accordingly; it was possible to apply the technique for quantitative analysis of NPF. DPV voltammograms obtained with increasing amounts of NPF showed a linear relationship between peak height and concentration, as shown in Fig 7. Regression

parameters were calculated according to ICH guidelines [35] as given in Table 1.

Validation of the proposed method

International Conference on Harmonization (ICH) guidelines[35]for method validation were followed for validation of the suggested method.

Linearity and range: Linearity relationship was verified over the concentration ranges indicated in (Table1) for DPV technique as shown in Fig. 7. Statistical analysis of the data gave high values of square correlation coefficient (R2) and small values of standard deviation (SD) and relative standard deviation(RSD) which figures out the low scattering of the points around the calibration graph and proved linearity of the method over the specified concentration range (Table 1).

Detection and quantitation limits: LODs and LOQs were calculated according to ICH guidelines [35]. LOD was determined by evaluating the lowest amount of analytes which can be detected but not necessarily quantities as exact values.LOQ was determined by establishing the lowest amount of analytes which can be quantitatively determined with suitable precision and accuracy. The results are summarized in Table 1.

Accuracy: To prove the accuracy of the proposed method, the results of the assay of NPF in pure form assessed by the proposed voltammetric method were compared with those obtained using the reported spectrophotometric method[5].Statistical comparison of the results obtained by the proposed method and those obtained by the reported method using student's t-test and F-test revealed no significant difference between the two methods as shown in Table 2.

Precision (repeatability and reproducibility): The precision of the method was investigated by performing three series of measurements with three different concentrations of NPF solution within same day to evaluate the intraday (repeatability). Also, three series of measurements were performed on two successive days (reproducibility of the same modified electrode) and using three consecutive newly modified electrodes (reproducibility of renewed modified electrodes). The results indicated high accuracy and precision of the proposed procedure and proved to be suitable for quality control of NPF as in Table 3.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by minor, but deliberate variations in method parameters. The robustness of the proposed method was investigated by constancy of the peak current with deliberate minor changes in the experimental parameters. The studied variables included the change in pH (± 0.2), the time considered before each measurement (10s±5s). These minor changes that may take place during the experimental operation did not affect the peak current intensity of the studied drug, indicating the reliability of the proposed method during normal usage.

Stability: The stability of the modified electrodes has been investigated. The peak current did not change after storage in air for 9 days. The modified electrode retained 98% of its initial response up to 1 month.

Specificity: Specificity was proved by comparing the voltammograms of the pharmaceutical preparation to that of the pure form, they were found similar. Besides, good results for the recovered concentrations of the pharmaceutical preparation prove specificity.

Analytical Application

Analysis of Nevaxal® eyedrops: The proposed method was successfully applied for the determination of NPF in its pharmaceutical dosage form. The obtained results are listed in Table 4 and showed non-significant differences with those obtained by reported method [5].

Assay of NPF in spiked human serum samples: The applicability optimized procedure of the proposed to quantitative determination of NPF concentration in human serum was successfully investigated; Acetonitrile and methanol were investigated asthe serum precipitating agents. The best effects were found with acetonitrile. So, acetonitrile was used for the subsequent studies. The measurements of NPF in serum samples were achieved as described in analysis of spiked serum samples. For the applicability of the suggested method to the human serum, the calibration equation was obtained in spiked serum. Parameters and validation data are displayed in Table 5.

Standard addition method: The standard addition method was applied to the commercial pharmaceutical formulation containing NPF. With the application of the standard addition method the mean percentage recoveries and their standard deviations for the proposed methods were calculated in Table 6. According to the obtained results good precision and accuracy were observed for this method.

CONCLUSION

This manuscript validates the direct measurement of NPF at two modified carbon paste electrodes in bulk powder, pharmaceutical dosage form and

biological fluid using two electrodes, the first electrode is the carbon paste modified with 10% w/w of 1-n-butyl-3-methylpyridinium hexa fluorophosphate ion crystal (10%BMH-CPE) and the second electrode is carbon paste modified with 10% w/w of each of multiwalled carbon nanotubes and 1-n-butyl-3-methyl pyridinium hexa fluorophosphates (CMWNT-BMH-CPE) applying differential pulse voltammetric techniques. Both electrodes were considerably stable up to 1 month when put in refrigerator. The electrodes preparation is easy and simple. The oxidation of NPF is catalyzed at pH 6.0, where both electrodes demonstrate highest sensitivity in voltammetric measurements of pure form, pharmaceutical dosage form and in spiked serum samples.

Table1: a) Regression data obtained from calibration curves NPF at (10% BMH-CPE) and (CMWNT-BMH- CPE) applying DPV technique.

Parameters	**(10% BMH-CPE)**	**(CMWNT- BMH-CPE)**
Linearity range (µg/ml)	1.67-11.69	1.67-13.36
Slope (µA)	0.634	0.819
Intercept	0.051	0.468
SE of Slope	0.102	0.118
SE of Intercept	0.109	0.235
Correlation Coefficient (r^2)	0.9997	0.9999
LOD (µg/ml)	0.118	0.125
LOQ (µg/ml)	0.394	0.415
Repeatability of the peak current [a] (SD)	0.025	0.033
Reproducibility of the peak current[a] (SD)	0.183	0.154
Repeatability of the peak potential [a] (SD)	0.023	0.062
Reproducibility of the peak potential [a] (SD)	0.149	0.168

[a] Obtained from an average of five experiments

Table 2. a) Accuracy of the proposed method for determination of NPF in its pure form using DPV technique.

Parameter	**(10% BMH -CPE)**		**(CMWNT-BMH -CPE)**		**Reported method [5]**
	Amount taken (µg/mL)	**R%**	**Amount taken (µg/mL)**	**R%**	
	3	99.33	3	100.67	
	6	100.17	6	100.17	
	9	100.22	9	99.78	
Mean ± SD		99.91±0.500		100.21±0.446	99.74±0.330
Variance		0.250		0.199	0.109
t-test	(ttab=2.78)	0.49		1.47	
Ftest	(Ftab=5.79)	2.29		1.83	

*each result is an average of three separate determinations

Table 3 a) Inter- and intra-days regression parameters for peak 1for determination of NPF.

	(10%BMH-CPE)			**(CMWNT-BMH-CPE)**		
parameter	**3.00**	**6.00**	**9.00**	**3.00**	**6.00**	**9.00**
Intraday	99.55±0.693	99.67±0.165	99.93±0.168	100.22±0.508	100.11±0.255	100.07±0.168
Interday	99.78±0.840	100.11±0.255	100.22±0.110	99.78±0.508	100.06±0.344	99.93±0.277
On same electrode						
On three different electrodes	100.00±0.883	99.89±0.535	100.00±0.485	99.67±0.884	100.00±0.440	100.11±0.330

Table 4: Application of the proposed and comparison methods for determination of NPF in pharmaceutical dosage form Nevaxal® eye drops

Dosage form Nevaxal® eye drops	**(10% BMH-CPE)**	**(CMWNT-BMH-CPE)**	**Reported. Method[5] (R%)**
Mean* ± SD	99.94±0.403	100.11±0.639	99.47± 0.330
Variance	0.162	0.408	0.109
t-test	2.02	1.15	(ttab=2.23)
F-test	1.49	3.74	(Ftab=4.88)

*average of nine determinations

Table 5: Appraisal of the accuracy and precision of the proposed method for determination of NPF in spiked serum samples

parameter	**(10% BMH-CPE)**	**(CMWNT-BMH-CPE)**
(mean ± SE [a])	99.74±0.251	99.75±0.177
SD[b]	0.434	0.307
variance	0.188	0.094

SE [a]: Standard error of three replicates of three different concentrations
SD[b]: Standard deviation of three replicates of three different concentrations

Table 6: Appraisal of the selectivity of the proposed method for determination of NPF by applying standard addition technique:

parameter	**(10% BMH-CPE)**	**(CMWNT-BMH-CPE)**
(mean ± SE [a])	99.64±0.074	100.11±0.455
SD[b]	0.128	0.788
variance	0.016	0.621

SE [a]: Standard error of three replicates
SD[b]: Standard deviation b Average of three replicate determination.

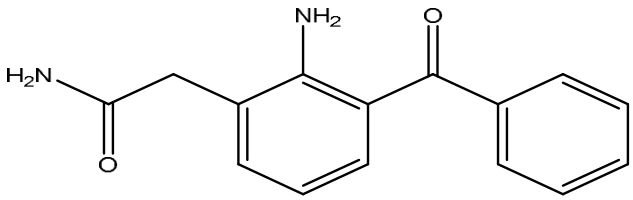

Figure 1. Nepafenac (NPF)

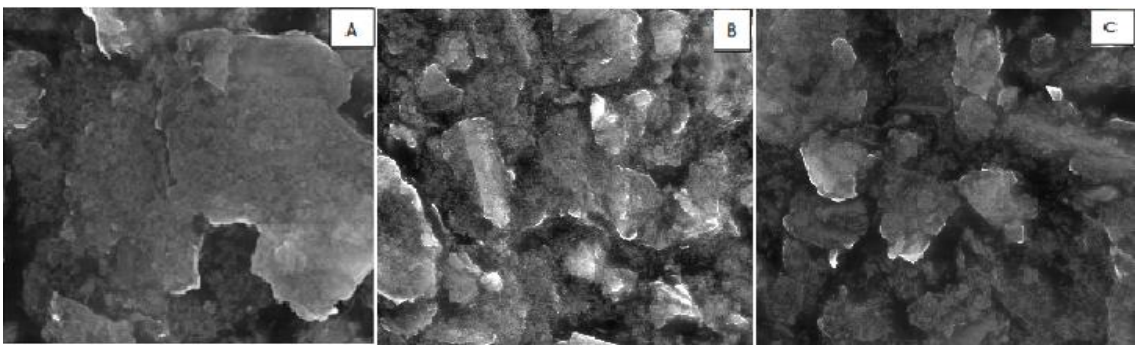

Figure. 2. SEM micrographs (magnification 10,000 x)for : (A) (CPE), (B) (BMH -CPE) and (CMWNT-BMH-CPE).

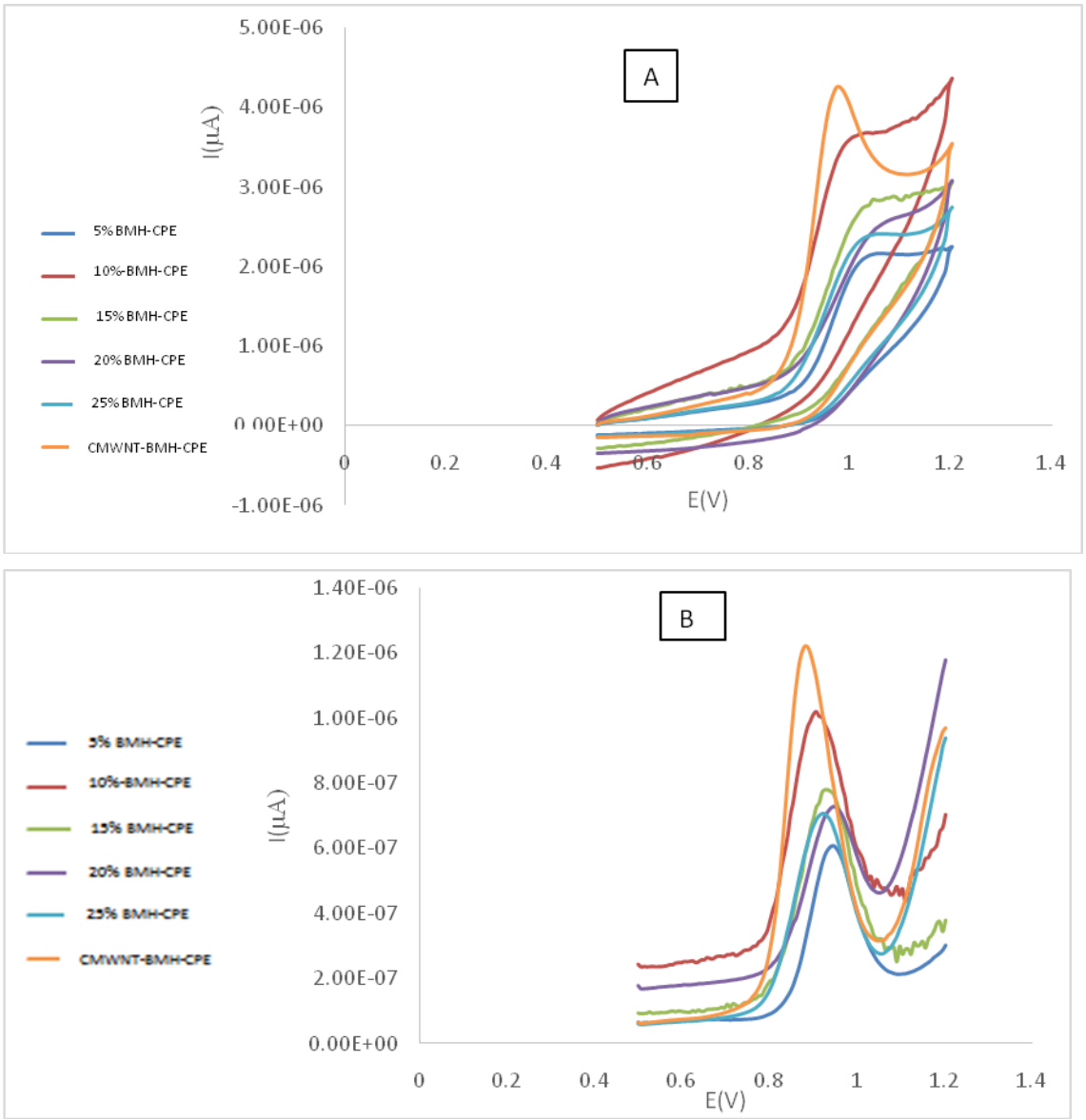

Figure 3 :(A) Cyclic voltammograms and (B) Differential pulse voltammograms (DPV) of 10 µg/mL NPF in BR buffer pH 6.0 at a scan rate 100 mV s-1 at different electrodes [different ratios of BMH-CPE

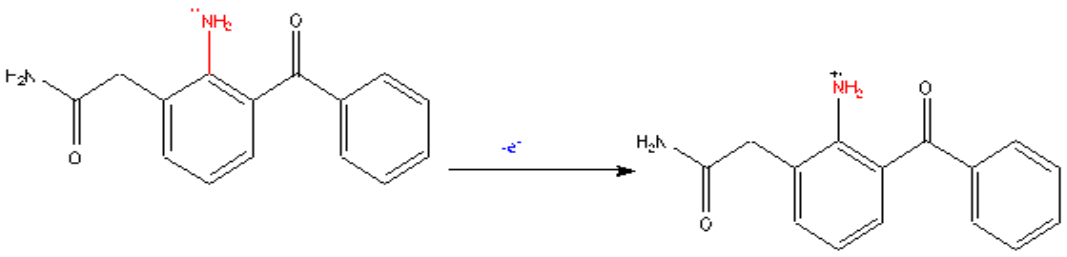

Figure 4. Proposed oxidation mechanism

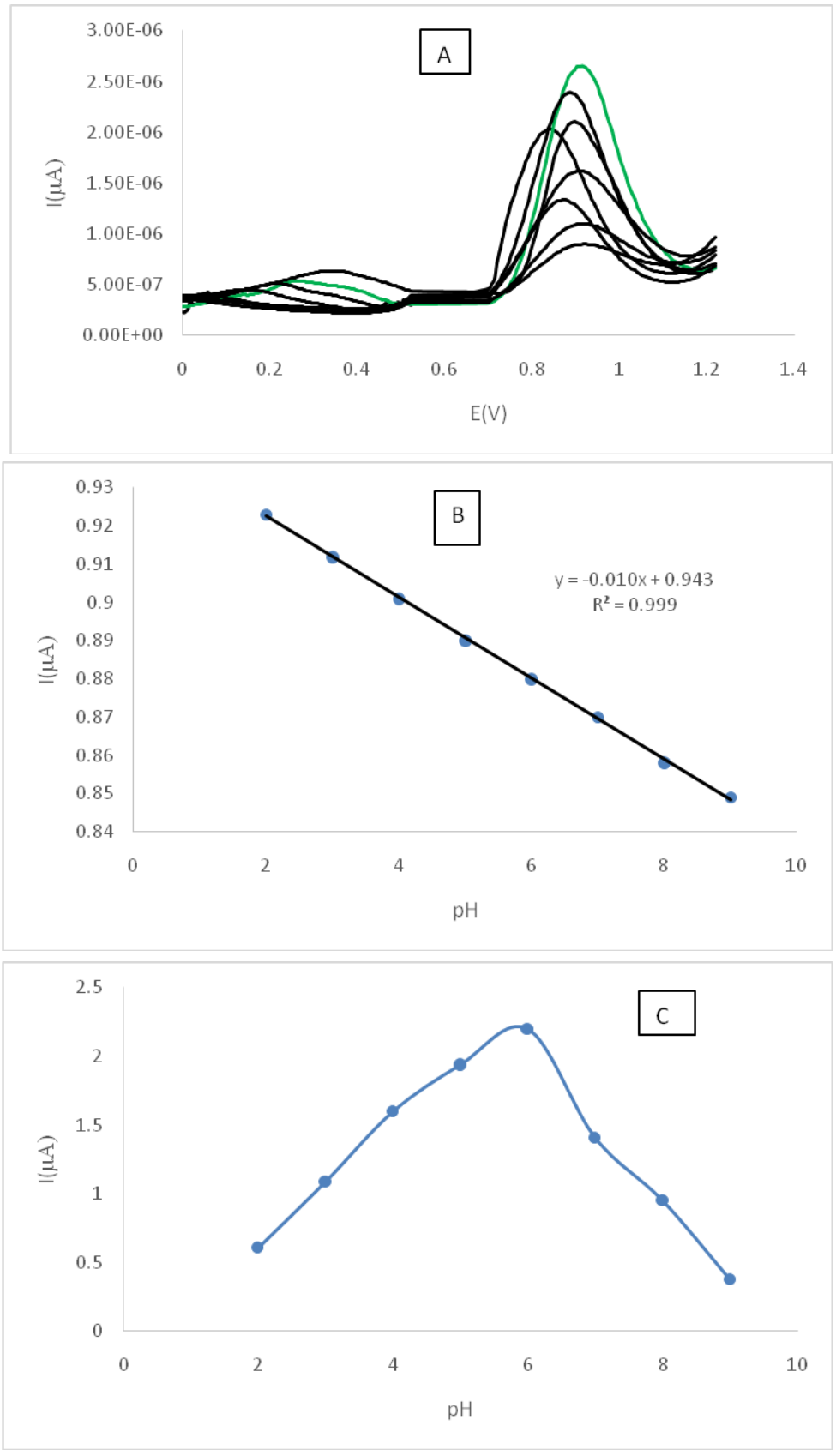

Figure 5. A) Differential pulse voltammetric response of 20 µg/mL NPF at (CMWNT-BMH-CPE) in 0.04 M B-R buffers of different pH values. B) Comparison between the anodic peak response and potential and C) Comparison between the anodic peak response and peak currents at different pH values

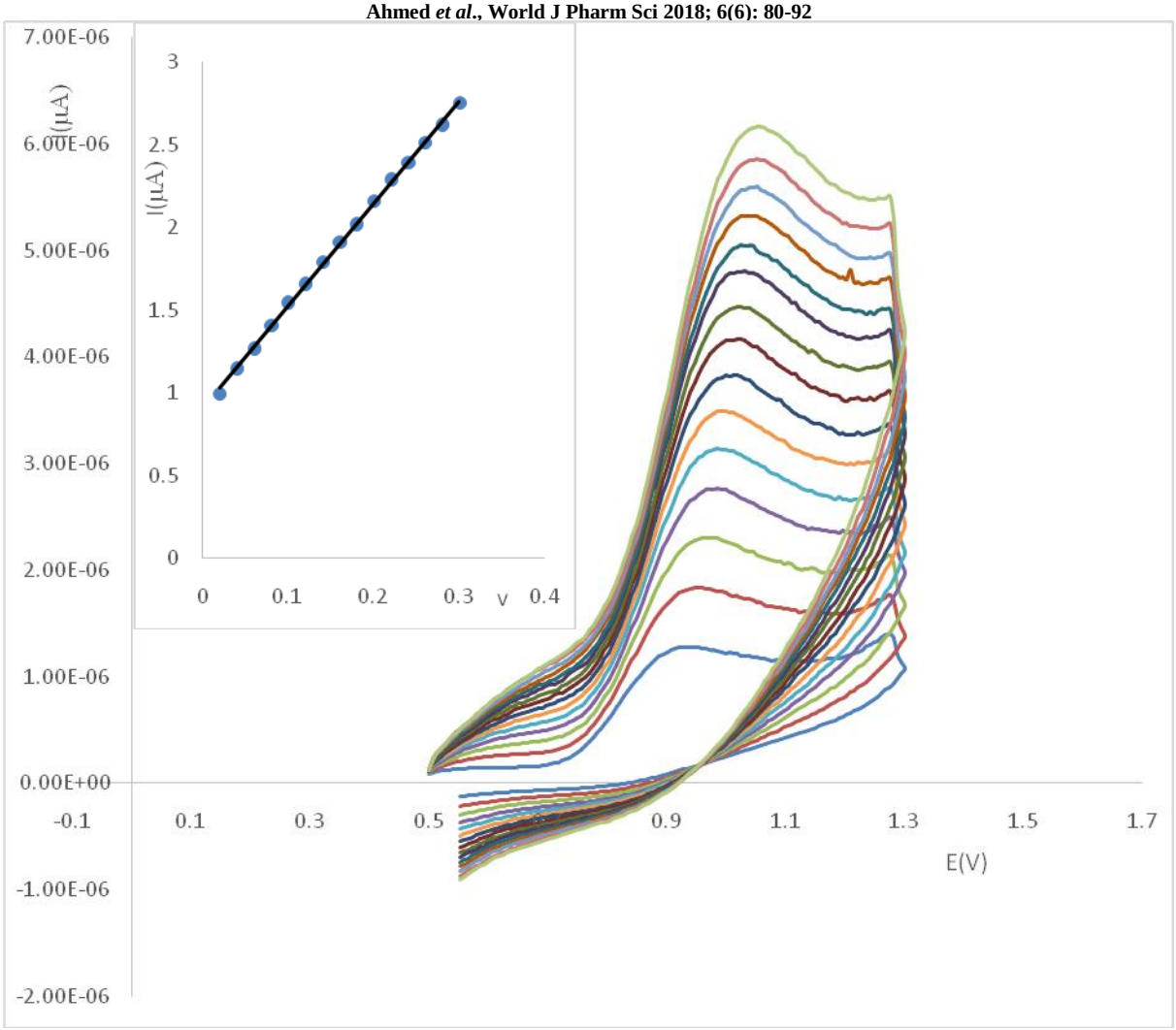

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Figure 6. (A) Scan rates on the current response of 10 µg/mL NPF at (CMWNT-BMH- CPE) in 0.04M B–R buffer (pH 6.0) at scan rates (inner to outer) of 20 to 300 mV s^{-1}. Inset: Plots of peak currents vs. the scan rate

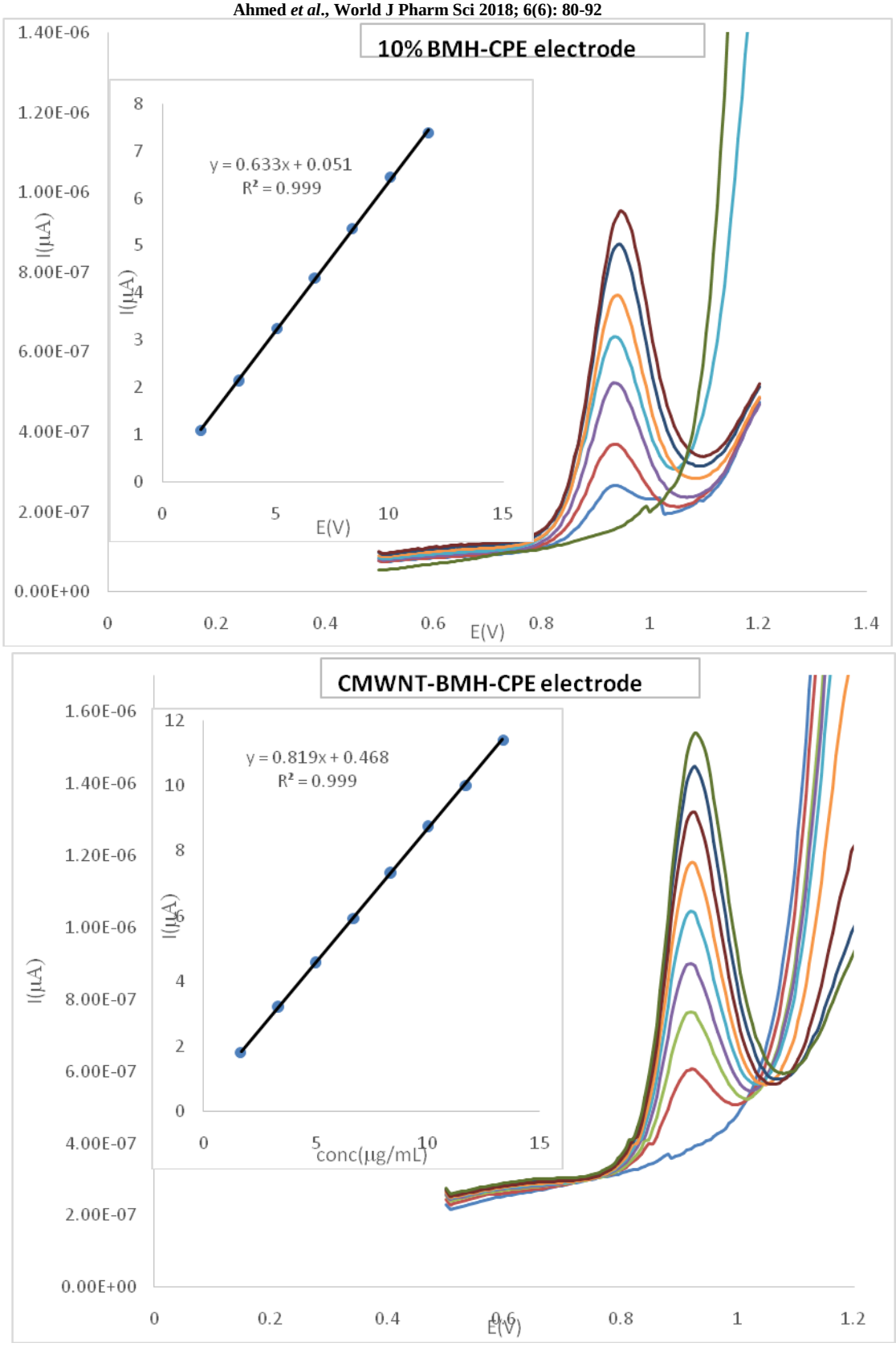

Figure7. DPV sweeps and calibration curves of NPF at (10%BMH-CPE) and (CMWNT BMH-CPE) in 0.04 M B-R buffer pH 6.0 and scan rate 100 mVs^{-1}

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