Talanta 108 (2013) 30–37

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

AuNPs-poly-DAN modified pyrolytic graphite sensor for the determination of Cefpodoxime Proxetil in biological fluids

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ARTICLE INFO

Article history:
Received 11 January 2013
Received in revised form
15 February 2013
Accepted 18 February 2013
Available online 4 March 2013

Keywords:
Cefpodoxime Proxetil
Pyrolytic graphite
1,5-Diaminonapthalene
Gold nano-particles

ABSTRACT

A sensitive and selective electrochemical method for Cefpodoxime Proxetil (CP) determination has been developed by incorporating gold nanoparticles (AuNPs) onto the poly-1,5-diaminonapthalene layer (p-DAN) coated pyrolytic graphite. The modified sensor was characterized by X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM). The sensor exhibited an effective catalytic response towards oxidation of CP with excellent reproducibility and stability. The peak current of CP was found to be linear in the range of 0.1–12 μM and detection limit and sensitivity of 39 nM ($S/N=3$) and 4.621 μA μM^{-1}, respectively, were observed. The method was successfully applied for the determination of CP in pharmaceutical formulations and human urine samples. The common metabolites present in human urine such as uric acid, ascorbic acid, xanthine and hypoxanthine did not interfere in the determination. A comparison of the results obtained by using developed method with high performance liquid chromatography (HPLC) indicated a good agreement. The method is simple, sensitive, rapid and precise and is useful for the routine determination of CP in pharmaceutical dosages and biological samples.

1. Introduction

Cefpodoxime Proxetil (CP), (6R, 7R)-7-{[(2Z)-2-(2-amino-1, 3-thiazol-4-yl)-2-methoxy imino-acetyl]amino}-3-(methoxy-methyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2 carboxylic acid, is a semi-synthetic beta-lactum antibiotic belonging to the third generation of cephalosporin group [1,2]. CP is a prodrug and it is hydrolyzed into its parent moiety cefpodoxime acid (CA) by specific cholinesterase enzyme in the intestinal wall/plasma to exhibit its antibiotic activity [3]. It shows broad spectrum antimicrobial activity against several microorganisms and its antibacterial action is suggested by binding to specific penicillin-binding proteins located inside the bacterial cell wall [4]. It is used orally for the treatment of mild to moderate respiratory tract infections, gonorrhoea and urinary tract infections and also in the treatment of skin infections, acute media otitis, pharyngitis and tonsillitis [5,6]. CP is absorbed orally throughout the gastrointestinal wall and shows about 50% bioavailability after the administration of CP as a 132 mg tablet (equivalent to 100 mg of cefpodoxime). The low bioavailability of CP is attributed to its poor water solubility and pre-absorption luminal metabolism into CA by the action of digestive enzymes [7–9]. It has been observed that CP is a well-tolerated antibiotic but it is not suitable for patients allergic to metabolites of CP. Adverse effects of this drug include maculopapular rash, bronchospasm, exfoliative dermatitis, Steven Johnson syndrome and anaphylaxis. Overdose of CP is associated with nausea, vomiting, flatulence, oral candidiasis, epigastric distress and diarrhoea [10]. The chemical structure of CP is presented in Scheme 1.

The broad spectrum clinical use of CP triggered our interest to develop a sensitive and rapid method for CP determination in pharmaceutical samples and human biological samples. Various techniques have been developed for the evaluation of cephalosporins in body fluids and dosage forms, which include spectrophotometric [11,12], fluorometric [13] and chromatographic techniques [14–16]. Although sensitivity and detection limit of CP determination have been improved in these techniques, these are rather expensive and require time consuming methods prior to analysis. Therefore, a simple and easy determination of CP in drug dosage forms, human urine and serum is desirable without time consuming extraction and separation steps with ease of analysis. In recent years, electroanalytical methods have attracted much attention of researchers towards the determination of drugs and various cephalosporins in dosage forms and biological fluids due to their high sensitivity and selectivity [17,18]. Many electrochemical methods have been described in literature concerning the reduction behaviour of CP using dropping mercury or hanging drop electrodes [19–21]. As mercury is toxic and causes environmental threat,

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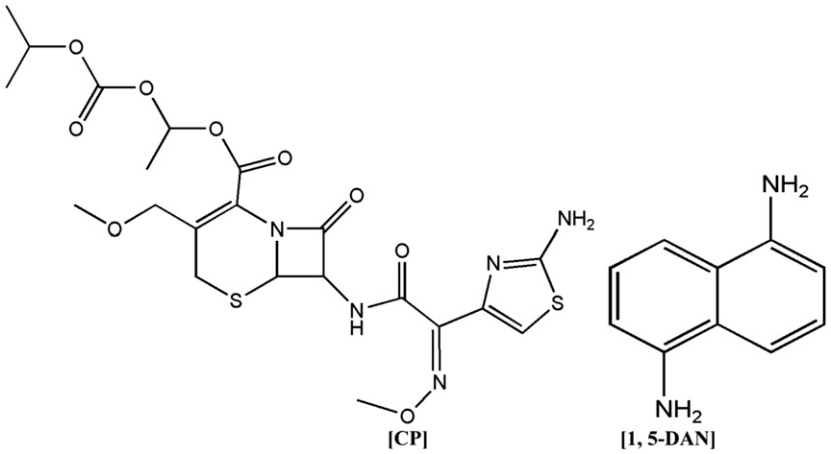
http://dx.doi.org/10.1016/j.talanta.2013.02.044



Scheme 1. Chemical structure of [CP] and [1,5-DAN].

solid electrodes have also been used to study the oxidation of cephalosporin group antibiotics [22–24]. Till date, no oxidation studies on the determination of CP have been reported at solid electrodes. Therefore, the aim of this study is to develop a simple electroanalytical method for the determination of this drug in pharmaceutical dosage forms and biological fluids.

Conductive polymers have continued to be the major concerns during the past decade due to their potential applications in battery electrodes, electrochromic devices and electroluminescent devices [25]. Application of conductive polymers in electrochemical sensors has been extensively increased due to their advantages, such as possibility of one step synthesis on different substrates with good stability, reproducibility and low cost. Recently, organic conductive polymers have been used to prepare chemically modified electrodes for highly sensitive and selective analysis of tetracycline and β-lactum antibiotic, amoxicillin [26,27]. Among organic compounds, aromatic amino compounds have gained particular attention for their capability to provide polymer coating on metallic or carbon electrode by electrooxidative polymerization. As poly-1,5-diaminonaphthalene (p-DAN) is found to have versatile applications in the construction of chemically modified sensors, it has been confirmed as a promising candidate to form polymer–drug conjugate for drug delivery purposes [28,29]. In the present paper we report the electropolymerization of 1,5-diaminonapthalene (1,5-DAN) at the edge plane surface of pyrolytic graphite (EPPG). As gold nanoparticles (AuNPs) have attracted substantial interest in electrode modification due to their capability of enhancing the electrode conductivity, which improves sensitivity and selectivity [30–32], incorporation of AuNPs onto p-DAN has also been carried out to fabricate the sensor. To the best of our knowledge, electrochemical oxidation of CP is reported for the first time involving low cost conducting polymer modified sensor. The electrochemical method presented in this work is a promising substitute to the frequently reported chromatographic, photometric and other analytical methods due to its simplicity, rapidity, reliability and low cost of analysis. The application of the proposed method has been demonstrated by the determination of CP in biological samples and pharmacological formulations and results have been successfully validated.

2. Experimental

2.1. Apparatus

All the voltammetric experiments were performed with a computerized Bio analytical system (BAS, West Lafayette, USA) CV-50 W voltammetric analyzer. A conventional single compartment three electrode glass cell equipped with EPPG/p-DAN/AuNPs sensor as the working electrode, Ag/AgCl (3 M NaCl) reference electrode (BAS Model MF-2052 RB-5B) and a platinum wire as the counter electrode was used. The pH of the phosphate buffers was measured using a digital pH metre (model CP-901). Pyrolytic graphite pieces were received as a gift from Pfizer Inc., New York, USA. A Field Emission Scanning Electron Microscopy (FE-SEM) instrument (JEOL, JSM-7400) was used to characterize the surface of the sensor. X-ray Photoelectron Spectroscopy (XPS) measurements were carried out using a VG scientific ESCA lab 250 XPS spectrometer coupled with a monochromated Al K-source having charge compensation, at Pusan National University, Busan (S. Korea). HPLC studies were carried out on a Shimadzu (LC-2010 HT) equipped with C_{18} reverse phase column. Mobile phase used was a mixture of 20 mM phosphate buffer (pH 7.2) and acetonitrile in the ratio of 62:38 at a flow rate of 1 ml/min and the absorbance of the eluent was monitored at 235 nm.

2.2. Reagents and materials

CP was purchased as a gift from Alkum Drugs and Pharmaceuticals Ltd., Haridwar (India). Phosphate buffers of different pH were prepared according to Christian and Purdy [33]. 1,5-DAN and hydrogen tetrachloroaurate ($HAuCl_4$) were purchased from Sigma-Aldrich. Perchloric acid ($HClO_4$) was purchased from Rankem Chemicals, Delhi. CP containing tablets manufactured by different companies were purchased from the local market of Roorkee. All other solvents and reagents used in the experiment were of analytical grade. Double distilled water was used throughout the experiments.

2.3. Fabrication of AuNPs-pDAN on the surface of EPPG

Prior to modification of edge plane surface of pyrolytic graphite (area 3 mm^2), it was rubbed on an emery paper (P-400) and then rinsed thoroughly with double distilled water and dried. p-DAN film was then grown in solution of 1 M $HClO_4$ containing 10 mM 1,5-DAN. Polymerization was carried out by cycling the potential between -0.1 and $+1.0$ V vs. Ag/AgCl (in saturated NaCl) at a scan rate of 100 mV s^{-1} for 50 scans. After the stable polymer film was prepared on the surface, it was rinsed with distilled water carefully in order to remove soluble products as well as monomer of 1,5-DAN before it was subjected to further experiments.

Fig. 1 presents a comparison of the XPS survey spectrum of unmodified and p-DAN modified surface of pyrolytic graphite.

Two sharp peaks were noticed in the unmodified surface at 299.1 and 540.3 eV corresponding to carbon and oxygen. After polymerization of DAN, a sharp peak at about 398.9 eV appeared, which was absent in the unmodified surface (Fig. 1), due to the NH_2 group which was present in the polymer backbone. This confirmed that p-DAN has been successfully deposited at the surface of pyrolytic graphite. Incorporation of gold nanoparticles on the surface of polymer coated pyrolytic graphite was then made by using cyclic voltammetry. For this, the potential from -0.8 to $+0.4$ V was scanned at a scan rate of 50 mV s^{-1} for 20 cycles in 1 mM $HAuCl_4$ solution as described in literature [34]. The electrode was then taken out, washed well with distilled water and dried under the flow of nitrogen. The XPS spectrum after AuNPs deposition on p-DAN modified pyrolytic graphite exhibited two additional peaks at 83.4 and 86.8 eV corresponding to Au4f as shown in Fig. 1. FE-SEM images of unmodified pyrolytic graphite surface (PGS), p-DAN modified PGS and AuNPs/p-DAN modified PGS are presented in Fig. 2, and clearly show the deposition of polymer and nanogold clusters at the EPPG surface. The polymer coated gold nanoparticles modified sensor was then ready for use and denoted as EPPG/p-DAN/AuNPs.

2.4. Experimental procedure

CP is insoluble in water; hence, stock solution of CP (1 mM) was prepared by dissolving the required amount of CP in a mixture of methanol and water (1:4) using a stirrer for 30 min. For recording voltammograms, aliquots of the stock solution of CP were diluted with 2 ml of phosphate buffer of pH 7.20 (μ=1 M) and total volume was made to 4 ml. The optimized instrumental parameters for square wave voltammetry (SWV) were initial potential (E): 0 mV, final potential (E): 1200 mV, square wave amplitude (E_{sw}): 25 mV, step potential (E): 4 mV and square wave frequency (f): 15 Hz. All the potentials are reported with respect to Ag/AgCl reference electrode at an ambient temperature of 25 ± 2 °C. Some other experimental parameters such as deposition time and stripping potentials were also optimized using 6 μM CP at EPPG/p-DAN/AuNPs sensor. For this purpose modified sensor was dipped in CP solution for the time period varying from 10 s to 200 s in the same solution. Variation of deposition time showed that peak current increased with deposition time and reached a plateau after a period longer than 120 s; hence, deposition time 120 s was chosen as an optimum time. Using optimum deposition time, a potential was applied in the range 0.0–1.0 V, which indicated that maximum value of peak current was observed at 0.660 V. Stripping potential of 0.660 V was selected as an optimum for further experiments.

The surface of the EPPG/p-DAN/AuNPs was cleaned after each run using time base technique by applying a constant potential (-100 mV) for 60 s in buffer.

The human urine samples of patients undergoing treatment with CP were obtained from the Institute hospital after the clearance from the ethics committee of Indian Institute of Technology, Roorkee. The samples were obtained after 6 h of administration of CP tablet (50 mg). Urine sample of normal person received from the laboratory personnel was used as control. Urine samples were diluted two times with phosphate buffer of pH 7.2 prior to analysis.

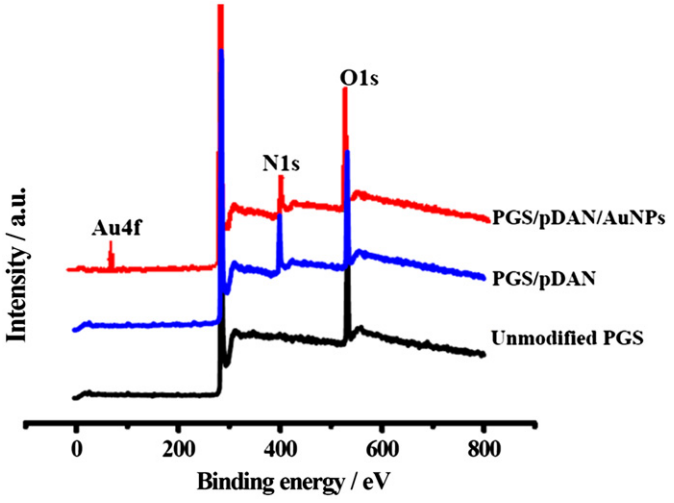


Fig. 1. Observed XPS survey spectrum of unmodified PGS, PGS/pDAN and PGS/pDAN/AuNPs.

3. Results and discussion

3.1. Fabrication of p-DAN/AuNPs film

Fig. 3 shows consecutive cyclic voltammograms recorded during the growth of p-DAN layer at the surface of pyrolytic graphite. In the first sweep towards positive potentials, the 1,5-DAN was oxidized exhibiting well-defined anodic peak ($E_p \sim 0.66$ V) corresponding to the oxidation of DAN monomer into cationic radical which is suppressed in subsequent potential cycles. A cathodic peak at 0.48 V was observed in the reverse sweep. During the early stage of growth two polymer anodic peaks at 0.32 V and 0.51 V and cathodic peaks at 0.15 and 0.47 V along with monomer oxidation peak at 0.68 V are observed due to the polymer film formation. The peak current of new peaks increased with increase in the number of potential scans, whereas peak current of peak at 0.68 V decreased with increase in the cycles. In the later stage of polymerization two anodic CV peaks

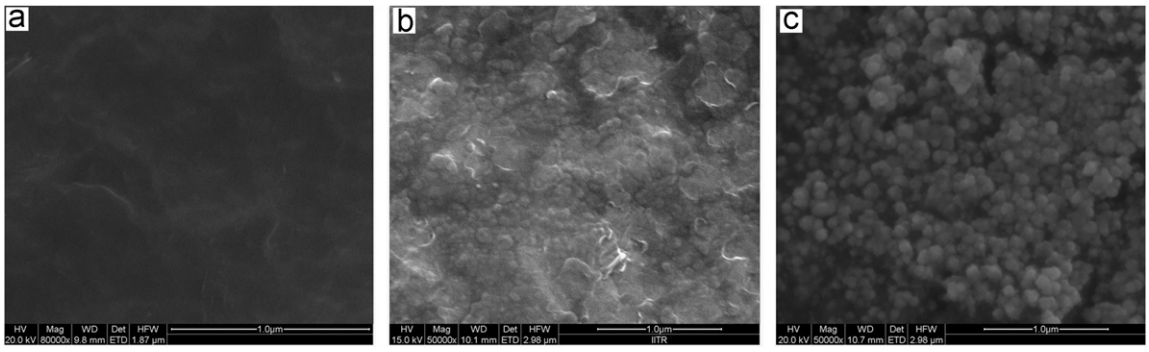


Fig. 2. Typical FE-SEM images of (a) unmodified EPPG, (b) EPPG/p-DAN and (c) EPPG/p-DAN/AuNPs.

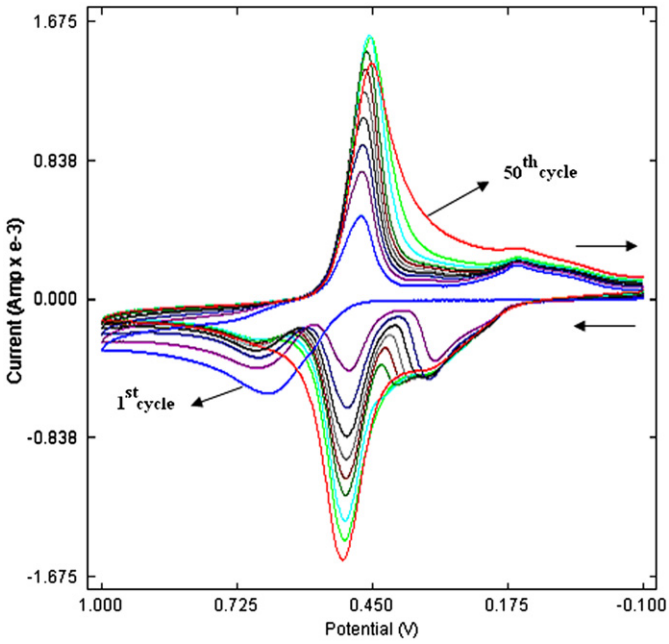


Fig. 3. A series of cyclic voltammograms recorded during fifty consecutive potential cycles between −0.1 and 1.0 V in 10 mM 1,5-diaminonaphthalene and 1 M $HClO_4$ at a scan rate 100 mV s^{-1} using EPPGE.

merged into one at about 0.52 V and finally in the 50th cycle two well-defined anodic and cathodic peaks were observed at 0.53 V and 0.45 V, respectively, related to the subsequent growth of polymer film and a uniform adherent polymer film was developed on the pyrolytic graphite surface. The CV results observed showed good agreement with the earlier reported electropolymerization studies of 1,5-DAN at gold disc electrode [35]. Electrochemical deposition of AuNPs on polymer coated EPPG surface provided nanostructured surface which exhibited excellent catalytic activity due to the unique properties of AuNPs such as increased surface area, good conductivity and biocompatibility [36].

3.2. Electrochemical response of p-DAN/AuNPs film

Electrochemical response of unmodified EPPG, EPGE/p-DAN and EPPG/p-DAN/AuNPs surfaces is examined by recording CV of K_3 $[Fe(CN)_6]$ and a comparison of three surfaces is presented in Fig. 4. A well-defined redox couple for Fe^{3+}/Fe^{2+} was observed at all the three surfaces. However, the peak currents for the redox couple increased in the case of EPPG/p-DAN/AuNPs (Fig. 4c) and the ΔE_p value decreased to 0.65 V showing more reversible nature of the redox couple at the modified surface. The effective surface areas after modification were also determined by recording cyclic voltammograms of 1 mM K_3 $[Fe(CN)_6]$ at different scan rates using 0.1 M KCl as supporting electrolyte. The surface area was calculated from the slopes of i_p vs. $v^{1/2}$ plots using Randles–Sevcik equation and found as 0.081, 0.102 and 0.174 cm^2 for unmodified EPPG, EPPG/p-DAN and EPPG/p-DAN/AuNPs surfaces, respectively. Thus, the EPPG/p-DAN/AuNPs sensor had an area more than double of the unmodified EPPG surface.

3.3. Cyclic voltammetry

The cathodic behaviour of CP has been reported at mercury electrodes earlier [20], however, no information is available concerning the electrooxidative behaviour of CP. Therefore, to study the anodic voltammetric behaviour of CP, first it was subjected to cyclic voltammetric study. Fig. 5 presents a cyclic

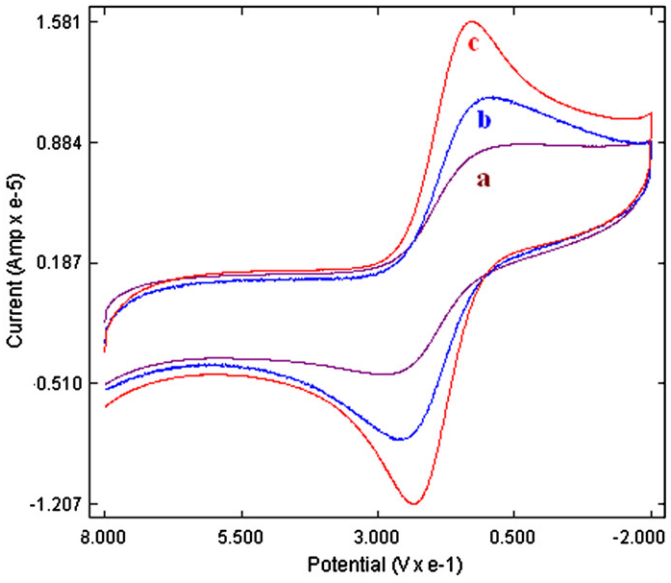


Fig. 4. Comparative cyclic voltammograms of 1 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl at (a) unmodified EPPG, (b) EPPG/p-DAN and (c) EPPG/p-DAN/AuNPs.

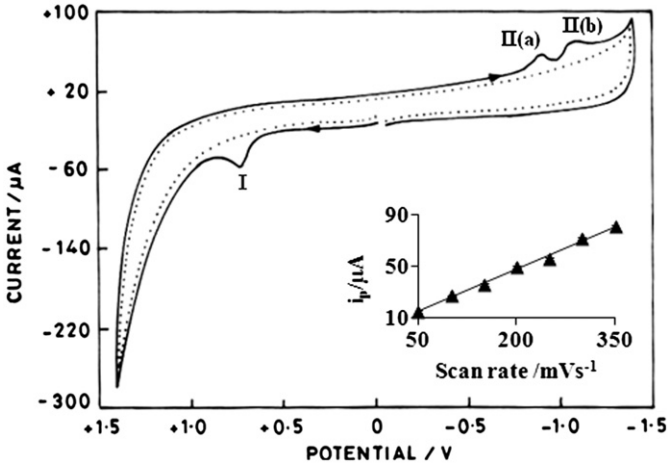


Fig. 5. Observed cyclic voltammogram for 6 μM CP at scan rate 100 mV s^{-1} at EPPG/p-DAN/AuNPs (——) and background phosphate buffer (….) at pH 7.20; inset is graph between i_p and scan rate (v).

voltammogram recorded for 6 μM CP using EPPG/p-DAN/AuNPs sensor at pH 7.2 at a scan rate of 100 mV s^{-1}. CP is irreversibly oxidized giving rise to a well-defined oxidation peak (I) at E_p (725 mV) when sweep is initiated in the positive direction and two reduction peaks at −950 mV (II_a) and −1110 mV (II_b) are observed in the reverse sweep. To confirm whether the reduction peaks are related to oxidation peak or are due to independent reduction of CP, CV was also recorded by initiating the sweep in the negative direction. In this case two reduction peaks were observed at −950 mV and −1110 mV and an oxidation peak was observed in the reverse sweep. Thus, it is concluded that CP can undergo oxidation as well as reduction. The anodic peak at 725 mV can be assigned to the oxidation of 2-amino group located on the thiazole ring in the side chain on C-7. Similar oxidation of amino group has been reported for cefixime, cefepime and related compounds [22,37]. As p-DAN layer exhibits two significant peaks at 0.53 V and 0.45 V in acidic medium and no peaks are observed while recording voltammograms in phosphate buffer of pH 7.20, it is concluded that the p-DAN layer shows redox behaviour only in acidic medium.

To ascertain the nature of the electrode reaction scan rate studies were performed in the range of 50–350 mV s^{-1}. The peak current of CP was found to increase with increase in the sweep rate and dependence of the anodic peak current on scan rate can be expressed by the following linear relationship:

$$i_p = 0.22[\nu] + 3.71$$

with a correlation coefficient of 0.995, where ν is the scan rate in mV s^{-1} and i_p is the peak current in μA. The linearity of i_p versus scan rate plot (inset of Fig. 5) indicated that oxidation of CP is adsorption controlled which was further confirmed by linearity of $i_p/\nu^{1/2}$ vs. log ν and log i_p vs. log ν plot. The following relation was observed for log i_p vs. log ν plot:

$$\log i_p = 0.859 \log \nu - 0.293$$

with a correlation coefficient 0.993. The slope value (>0.5) of log i_p vs. log ν plot further confirmed that oxidation of CP is followed by adsorption of CP at the electrode surface [38,39].

3.4. Square wave voltammetry

As square wave voltammetry (SWV) is considered to be a more sensitive technique than cyclic voltammetry, further studies are carried out using SWV. Square wave voltammograms were recorded for 6 μM CP at unmodified EPPG, EPPG/p-DAN and EPPG/p-DAN/AuNPs surfaces in phosphate buffer solution of pH 7.2 using the optimized parameters of SWV. On scanning the potential 0–1200 mV, a well-defined oxidation peak is obtained at potential 660 mV at EPPG/p-DAN/AuNPs. Under a similar condition, small bumps were observed at potential 720 mV and 733 mV at EPPG/p-DAN and unmodified EPPG, respectively (Fig. 6). The remarkable increment in peak current and decrement in oxidation potential proved that EPPG/p-DAN/AuNPs sensor has excellent electrocatalytic properties to enhance the rate of electrochemical process towards the oxidation of CP. Hence, EPPG/p-DAN/AuNPs sensor was used for further analytical studies of CP.

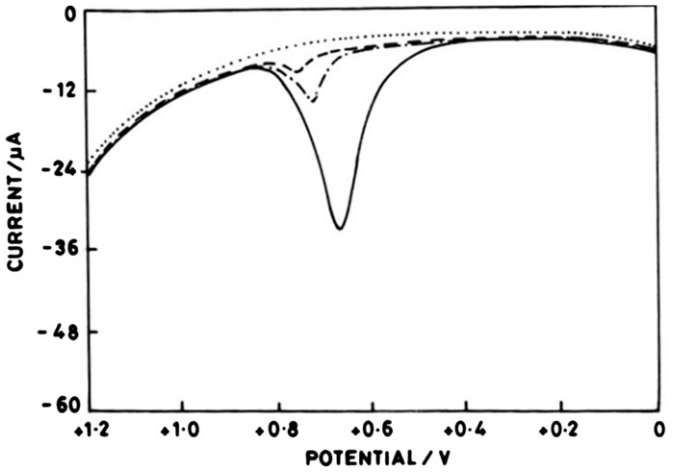


Fig. 6. Comparison of square wave voltammogram for 6 μM CP at bare EPPGE (– – –), EPPG/p-DAN (–●–), EPPG/p-DAN/AuNPs (——) and back ground phosphate buffer (…) at pH 7.20.

3.4.1. pH study

The pH of supporting electrolyte has a remarkable effect on the peak potential of the electrochemical species. The effect of pH of phosphate buffers was studied in the range 2.4–11.0 and it was found that the value of peak potential of CP shifted to less positive potential with increase in pH (Fig. 7a). The sharpness of peak and peak current value did not significantly change in the pH range 2.4–7.2, but the peak current decreased at pH > 7.2, probably due to the poor availability of protons. Hence, pH of the supporting electrolyte was kept constant at physiological pH 7.2 and all experiments were carried out using this pH. The E_p of CP shifted to less positive potential with increase in pH and the dependence of E_p on the pH of the supporting electrolyte at EPPG/p-DAN/AuNPs sensor can be described by the equation

$$E_p(\text{pH}2.4-11.0) = [-57.62\text{pH} + 1053]\text{V vs. Ag/AgCl}$$

with a correlation coefficient of 0.991. The observed value of the slope of dE_p/dpH indicated that an equal number of protons and electrons take part in the electrochemical oxidation of CP. This behaviour suggests that the oxidation mechanism of CP is closely related to the mechanism of other similar cephalosporin antibiotics [22,23].

3.4.2. Frequency study

The dependence of the anodic peak current of CP on the square wave frequency was studied in the range 5–40 Hz using EPPG/p-DAN/AuNPs sensor. The anodic peak current of CP was found to increase linearly with increasing square wave frequency in the range 5–40 Hz (Fig. 7b). The linear relationship between peak current and square wave frequency can be described by the equation

$$i_p = 1.319[f] + 9.49$$

having R^2 of 0.994, where i_p is the peak current in μA and [f] is the square wave frequency in Hz. The above voltammtric response further confirmed that the nature of electrode reaction is adsorption controlled [39].

3.4.3. Concentration study

The quantitative determination of CP is based on the dependence of the anodic peak current on the concentration of CP. Therefore, square wave volltammograms were recorded at different concentrations of CP in the range 0.1–20 μM using the EPPG/p-DAN/AuNPs surface as shown in Fig. 8. The values of the anodic peak current are obtained by subtracting the background current

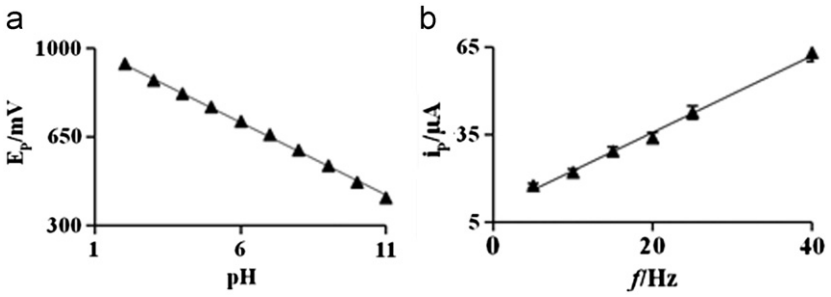


Fig. 7. (a) Effect of pH of supporting electrolyte on E_p at EPPG/p-DAN/AuNPs. (b) Variation of peak current (i_p) with square wave frequency (f) at EPPG/p-DAN/AuNPs.

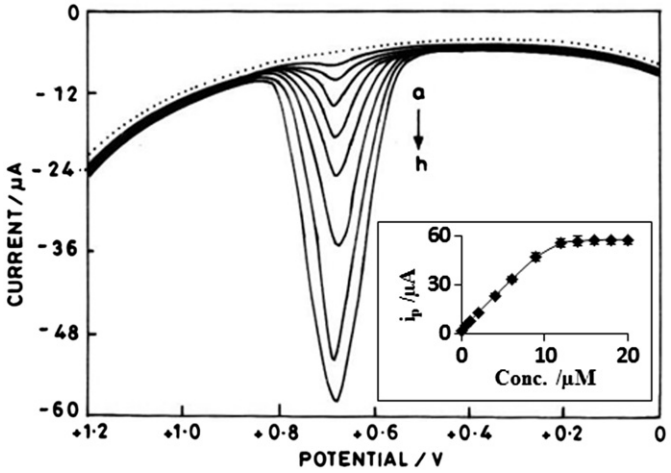


Fig. 8. Observed square wave voltammograms for background phosphate buffer (…) and increasing concentration of CP. Curves were recorded at (a)=0.1; (b)=0.5; (c)=1.0; (d)=2.0; (e)=4.0; (f)=6.0; (g) 9.0 and (h) 12.0 μM concentrations using EPPG/p-DAN/AuNPs in phosphate buffer of pH 7.2. Inset is the calibration plot between [C] and i_p.

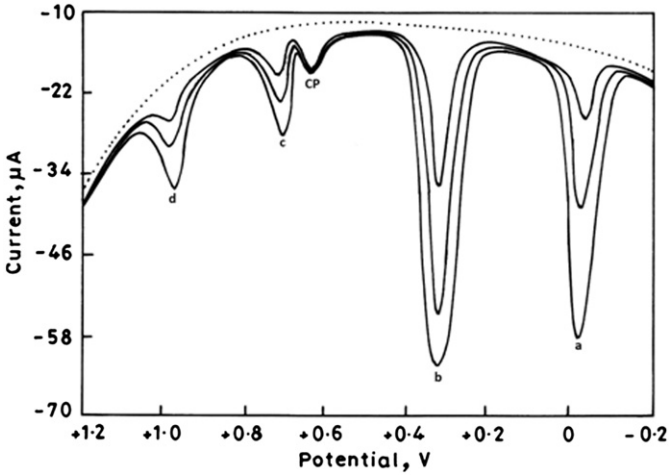


Fig. 9. Square wave voltammograms showing interference of ascorbic acid (peak a), uric acid (peak b), xanthine (peak c) and hypoxanthine (peak d) at fixed CP concentration (1 μM); dotted line shows background current of phosphate buffer.

and reported as an average of three replicate determinations. The peaks currents were found to increase with increase in the concentration range 0.1–12 μM of CP and become constant at higher concentrations. The plot of concentration vs. i_p was linear in the range 0.1–12 μM (inset of Fig. 8). The linear relationship in anodic peak current and concentration of CP can be expressed by the following regression equation:

$$i_p = 4.621[C] + 3.050$$

with a correlation coefficient of 0.990, where i_p is the peak current in μA and C is the concentration of CP in μM. The sensitivity of the proposed method is found to be 4.621 μA μM^{-1} and the detection limit was found to be 39 nM using the formula $3\sigma/b$ where σ is the standard deviation of the blank solution and b is the slope of the calibration plot. These results indicate that using EPPG/p-DAN/AuNPs sensor CP can be determined in biological fluids.

3.5. Stability and reproducibility of EPPG/p-DAN/AuNPs sensor

The long-term stability of the EPPG/p-DAN/AuNPs sensor was investigated by measuring the anodic current response for a fixed concentration of 1 μM CP over a period of 15 days. The modified sensor was used daily and stored in air. The experimental results revealed that the current response of CP deviated interday by ±2.1%, suggesting thereby, that EPPG/p-DAN/AuNPs sensor possessed excellent stability for the determination of CP.

To establish the intraday reproducibility of EPPG/p-DAN/AuNPs, consecutive repetitive determinations (n=10) of 1 μM CP were carried out at the same time and several measurements (n=6) were also made at an interval of 1 h each. The results indicated that the current response deviated during repetitive measurements by ±1.2% and intraday by ±1.9% exhibiting the excellent reproducibility of EPPG/p-DAN/AuNPs sensor for CP detection.

3.6. Interference study

Several metabolites present in urine or blood may alter the electrochemical signal of the sensor and consequently affect the selectivity of developed method. Uric acid, ascorbic acid, xanthine and hypoxanthine are common metabolites present in biological systems, which can interfere in the electrochemical response of CP. Hence, interference study was carried out at pH 7.2 by keeping the concentration of CP fixed at 1 μM and varying the amount of interferents up to 100-fold excess. In all these voltammograms, CP exhibited an anodic peak at 660 mV and some additional peaks were also observed at −10, 280, 790 and 970 mV corresponding to the oxidation of ascorbic acid, uric acid, xanthine and hypoxanthine, respectively (Fig. 9). It was found that there was no significant change in the anodic peak current response of CP up to 100-fold excess of each of the interferents. This proved that the developed method can be successfully applied for the determination of CP in human body fluids as well as in pharmaceutical preparations without facing any complexity due to the interferents.

Table 1
Determination of CP in pharmaceutical samples using EPPG/p-DAN/AuNPs sensor.

Sample	Stated content (mg)	Determined content[a] (mg)	Error (%)
Switch-50	50	49.62	−0.76
Monocef	100	99.14	−0.86
Switch-200	200	198.04	−0.98

[a] RSD for the determination was $< \pm 2.1\%$ for n=5.

3.7. Analytical utility

3.7.1. Pharmaceutical samples analysis

To demonstrate the applicability of the proposed method in drugs, three commercially available CP containing pharmaceutical samples, viz. Switch-50 (Alkem Lab Ltd., Baddi, Himachal Pradesh), Monocef (Aristo Pharma Pvt. Ltd., Daman, U.T.), Switch-200 (Alkem Lab Ltd., Baddi, Himachal Pradesh), were purchased from the local market of Roorkee. Solution was obtained by the dissolution of pharmaceutical samples in methanol and water (1:4) and the samples were subsequently diluted with buffer (pH 7.2) so that concentration of CP lies in the range of calibration plot. Square wave voltammograms were then recorded under identical conditions that were used for the concentration study. Keeping dilution factor in consideration concentration of CP in pharmaceutical samples was determined and the results were in good agreement with the labelled amount as shown in Table 1. The CP content for all the pharmaceutical samples were within an error range of −0.7% to 0.9% demonstrating the good accuracy of the developed method.

3.7.2. Real samples analysis

In order to examine the stability of CP in biological samples and establish the utility of the proposed method, the EPPG/p-DAN/AuNPs sensor was applied for the determination of CP in human urine samples of the patients undergoing treatment with CP. Prior to analysis, the urine samples were diluted 2 times with phosphate buffer to reduce the matrix complexity. Square wave volltammogram of patient urine sample is presented by curve a in Fig. 10 and a well-defined peak of CP is observed at ~662 mV in addition to a peak at 280 mV corresponding to the oxidation of uric acid. To confirm that the peak at 662 mV is due to the oxidation of CP, the sample was spiked with a known amount of CP and the peak current increased (Fig. 10 curves b and c). It was found that the peak current of CP increased on spiking CP, while uric acid peak current (at potential ~280 mV) remained constant. The concentration of CP in urine samples of patients before and after spiking was calculated by preparing a standard addition plot as shown in inset of Fig. 10. The absolute value of the *x*-intercept represents the concentration of CP in the urine sample of a patient undergoing treatment with CP and is found to be 3.32 μM. These results were validated in the form of statistical terms such as precision, RSD% and Bias%, which were found to be 0.054, 1.62% and 0.60%, respectively for $n=5$, proving the validity of the proposed method.

The recovery experiments were also carried out to check the stability of CP in human body fluids using EPPG/p-DAN/AuNPs.

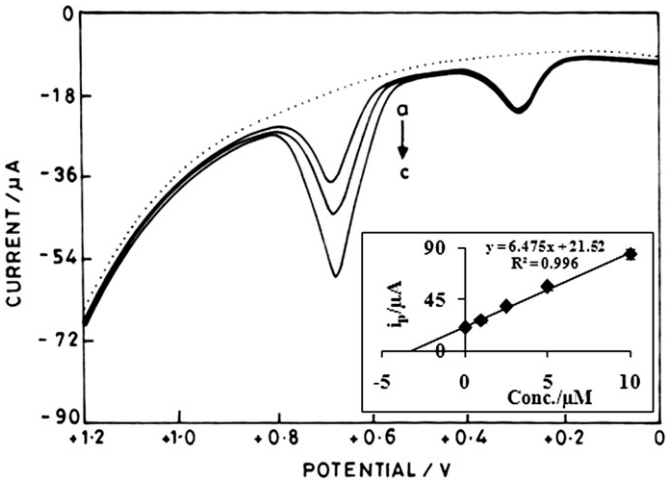


Fig. 10. Square wave voltammograms observed for determination of CP in the urine sample of patient (peak a) and after spiking (peaks b and c) with standard CP; dotted line shows blank at pH 7.20, and the inset is the standard addition plot of CP.

Table 2
Recovery analysis of CP in urine samples of healthy person at EPPG/p-DAN/AuNPs sensor.

Spiked amount (μM)	Detected amount[a] (μM)	Recovery (%)
Sample 1		
0.50	0.49	98.00
1.00	0.97	97.00
1.50	1.48	98.67
Sample 2		
0.50	0.48	96.00
1.00	0.98	98.00
1.50	1.49	99.33

[a] The R.S.D. value for CP determination was less than ± 3.4% for $n=3$.

Square wave voltammogram of urine sample of two healthy persons were recorded. The standard addition method was used for recovery experiments. Drug free urine samples were spiked with known concentration of standard solution of CP. The standard addition plot was used to calculate the concentration of CP and results obtained are summarized in Table 2. CP showed recovery in the range 101–99%, with relative standard deviation of ± 3.4% indicating good stability of CP in human biological samples.

3.8. Validation with HPLC

In order to prove the reliability of data obtained and validate the result of voltammetric determination of CP, HPLC method was used. For this purpose different concentrations of standard CP were analyzed using HPLC and a well-defined peak was obtained at retention time (R_t) ~2.869 min. The peak area under the peak was calculated and a calibration curve was obtained by plotting

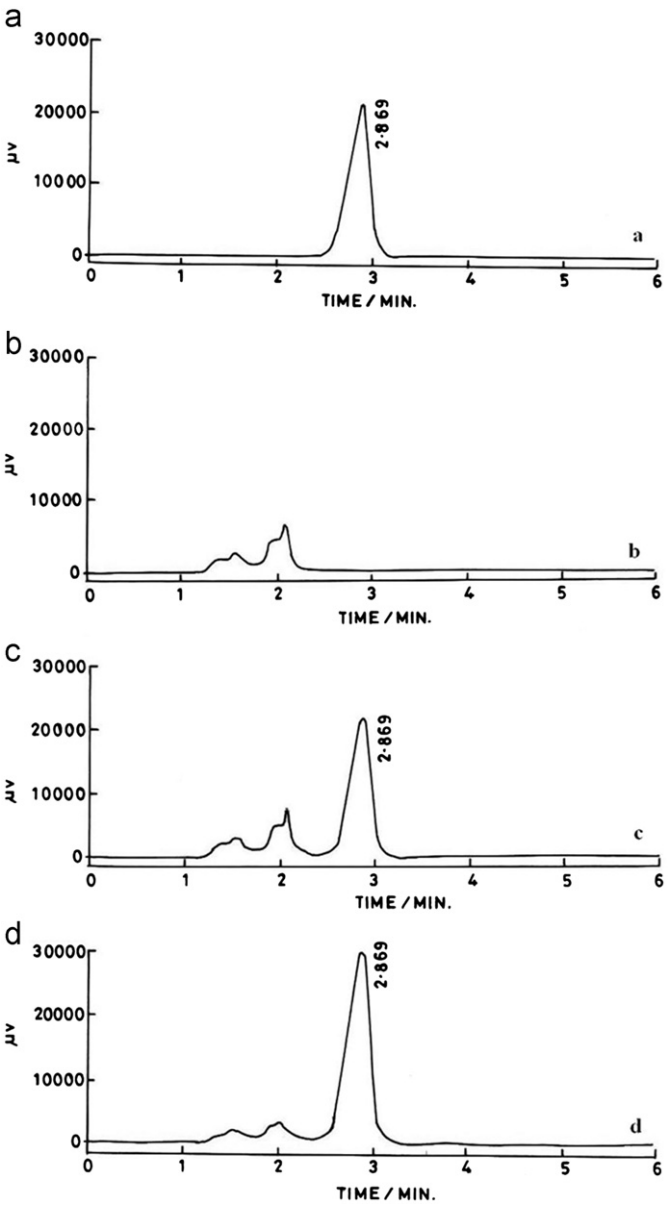


Fig. 11. Observed HPLC chromatograms of (a) standard CP, (b) control urine, (c) patient urine sample 1 treated with CP and (d) patient urine sample 1 after spiking with CP.

Table 3
HPLC validation of the results obtained for the determination of CP in human urine samples at EPPG/p-DAN/AuNPs sensor after 6 h of oral administration of CP.

Sample	Observed CP concentration (μM) determined by	
	EPPG/p-DAN/AuNPs	HPLC
1	3.32 (1.01%)	3.34 (1.21%)
2	3.34 (2.13%)	3.33 (1.76%)
3	3.92 (1.87%)	3.90 (3.76%)

The values in brackets represents the relative standard deviation for $n=3$ determination.

the peak area of the CP peaks against concentration. We obtained a linear calibration plot and finally the concentration of CP was determined in urine samples. Typical HPLC chromatograms observed for human urine sample of patients undergoing treatment with CP exhibited five peaks at $R_t \sim 1.400$, 1.549, 1.940, 2.068 and 2.869 min (Fig. 11). The peak at $R_t \sim 2.869$ is found to be due to CP, whereas, other peaks are due to the common metabolites present in urine samples. Concentration of CP in urine sample was calculated using the calibration plot of standard CP obtained from HPLC analysis. These results were compared with the results obtained from real sample analysis (Table 3). The results clearly indicated that the results obtained by the two methods are in good agreement confirming the accuracy of the present protocol. In addition, the HPLC method and electrochemical method were compared by performing student's *t*-test and *F*-test at significance level of 0.05. The *t*-values and *F*-values were observed as 0.012 and 1.091, respectively, which were smaller than the critical values of these observation, indicating strong evidence towards the null hypothesis (i.e. results of both treatments do not differ from one another). Values of probability factor for *t* and *F* tests were 0.991 and 0.479, respectively, which were much higher than that of significance level, further confirming the high probability of getting these results. These observations provide a big support to claim the accuracy of both methods.

4. Conclusions

The proposed work provides an extremely sensitive and selective electroanalytical method for the determination of CP in pharmaceutical samples as well as in biological fluids using EPPG/p-DAN/AuNPs sensor. The fabricated sensor was characterized by XPS and SEM studies. p-DAN modified pyrolytic graphite having AuNPs not only significantly increased the anodic peak current but also shifted the peak potential of CP to lower potentials as compared to the unmodified EPPG. The enhanced eletrocatalytic activity is attributed to the conductivity of polymer coated electrode and increased surface area due to gold nano-particles. Unique properties of AuNPs, such as stability, sensitivity and ability of electrocatalysis, have been shown to be a versatile tool to construct sensors due to which AuNPs have been used by several earlier researchers [40,41]. The combination of conductive polymer and AuNPs allowed the successful determination of CP with a detection limit of 32 nM. This detection limit is much lower than that reported in literature in which determination of CP is based on reduction [19,20]. The selectivity of the present method was also investigated in the presence of other substances present in the complex matrix. The application of the developed method for the determination of CP in urine of patients undergoing treatment with CP has also been carried out and the results are compared with HPLC. An excellent correlation between the two techniques is observed indicating thereby that the proposed work is sensitive and selective for the determination of CP.

Acknowledgement

Authors (BA) and (SKY) are thankful to CSIR, New Delhi, for awarding Senior Research Fellowship and Junior Research Fellowship respectively. Thanks are due to Dr. Pranjal Chandra, PNU, Busan, South Korea for help in recording XPS of samples. Financial assistance for this work was provided by the Council of Scientific and Industrial Research, New Delhi, vide Grant no. 01/2419/10-EMR-II.

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