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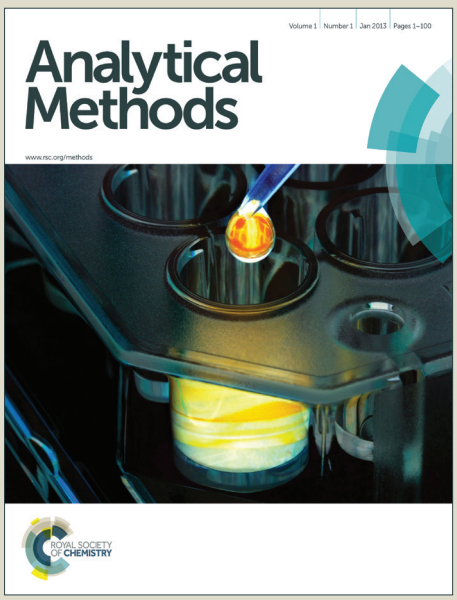

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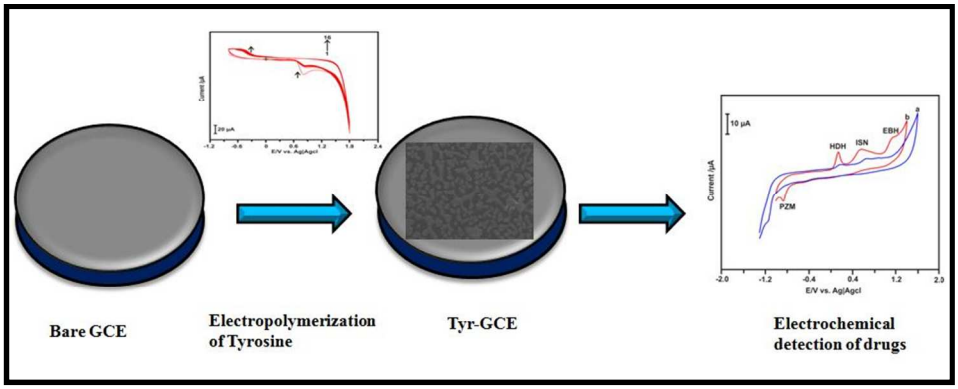

Electrochemical determination of selected antihypertensive and antituberculosis drugs at tyrosine modified electrode

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Abstract

A simple and sensitive electrochemical method was proposed for the determination of hydralazine hydrochloride (HDH), isoniazid (ISN), ethambutol hydrochloride (EBH) and pyrazinamide (PZM).For the first time tyrosine (Tyr) modified glassy carbon electrode (GCE) was employed for the determination of HDH, ISN, PZM and EBH by differential pulse voltammetric (DPV) technique. The proposed modified electrode showed strong electrocatalytic activity towards the above mentioned drugs with higher peak enhancement than that of unmodified electrode. The practicality of the proposed electrode for the detection of HDH, ISN, PZM and EBH in human urine and blood serum samples was successfully demonstrated using DPV technique. Applicability of the proposed method was verified with commercially available pharmaceutical tablets and the obtained results were in good agreement with the claimed label amounts of the tablets. From these results it was clear evident that the proposed electrode showed good catalytic activity towards the HDH, ISN, PZM and EBH. In addition, this method could be used for accurate detection of HDH, ISN, PZM and EBH in clinical and pharmaceutical industries in future.

Key words: Tyrosine. Hydralazine. Isoniazid. Ethambutol. Pyrazinamide

1. Introduction

Tuberculosis (TB) is caused by the Mycobacterium tuberculosis, causes leading death rate of adults worldwide and the number is decreasing since 2006[1, 2]. In order to preventing the development of tuberculosis, isoniazid (ISN), pyrazinamide (PZM) and ethambutol hydrochloride (EBH) were widely prescribed medicine for the treatment of tuberculosis, along with the fixed dose combination of rifampicin[3, 4]. Another foremost causing death rate in the earlier of 19th century was congestive heart failure due to hypertension [5] and the death was reduced in the 1950s and 1960s by introduction of anti hypertensive drugs. Hydralazine hydrochloride (HDH) is widely prescribed in the treatments of hypertension and congestive heart failure diseases [6]. The continuous intake of HDH induces various toxic reactions and causing dermatitis problems. Similarly overdose of anti-tuberculosis drugs causes severe side effects; particularly excess intake of ISN and PZM causes hepatic failure, further leading to fatal condition due to the excess formation of hydrazine during the INZ metabolism[7, 8]. In addition, overconsumption of EBH causes ophthalmic problems[9, 10]. Hence, due to the above facts and severity of the drug side effects; it is an essential to assess the HDH, ISN, EBH and PZM concentration levels in body fluids for effective therapeutic treatments. Hence, it is very important to develop a simple, reliable and sensitive method for the determination.

To date, few analytical methods were previously reported for EBH determination such as electrochemical method[10] liquid chromatography[11, 12], gas chromatography[13], capillary electrophoresis[14] and spectrophotometry[15]. Similarly for the detection of ISN, various analytical methods were reported earlier, such as amperometric method,[16] spectrophotometry[17], chemiluminescence[18] and electrochemical method[19]. Further for PZM detection few reported methods were available such as capillary electrophoresis, [20] electrochemical methods [21, 22]liquid chromatography-tandem mass spectroscopy (LCTMS) [23], UV-Vis spectrophotometry[24], chromatography[25] and square-wave polarography[26]. As well, HDH were previously reported by various methods such as electrochemical method [27], flow injection chemiluminescence[28], mass spectrometry (MS) [29], high-performance liquid chromatography (HPLC)[30, 31], colorimetry[32], gas chromatography [33] and spectrometry. [34]

Though, several methods were available for the determination of HDH, ISN, PZM and EBH. But electrochemical methods were attracted considerable attentions recently for the detection of various pharmaceutical drugs, owing to their simplicity with cost effectiveness, portability and high sensitivity.[35]In recent years poly amino acid modified electrodes playing a significant role in the pharmaceutical drug detection, due to the excellent electrocatalytic properties,[36] further various amino acid modified electrodes have been prepared in recent years by chemical and electrochemical methods for electrochemical sensor applications[37]. Among them Tyrosine (Tyr) modified

electrodes were playing a vital role in the electrochemical sensor due to its excellent film stability and electrocatalytic activity. Further, Tyr is a semi-essential amino acid and playing a vital role as a precursor for the neurotransmitters including nor-epinephrine and epinephrine[38, 39].

Herein, we report the fabrication of Tyr-GCE for the determination of HDH, ISN, PZM and EBH by DPV. Further, this sensor was effectively employed for the detection of the above mentioned drugs in commercially available tablets and real samples to demonstrate the practicality and applicability of the proposed method. Further, we investigated the morphological characterization of the modified GCE and electrochemical behaviors at modified electrode. To the best of our knowledge, till date no reports were available for Tyr modified GCE to determine the HDH, ISN, EBH and PZM in human blood serum, urine and pharmaceutical tablet samples.

2. Experimental

2.1 Chemicals

Isoniazid, Pyrazinamide, Ethambutol hydrochloride, Hydralazine hydrochloride and DL-Tyrosine were procured from Sigma-Aldrich. The supporting electrolyte used for all the experiments was pH 7 phosphate buffer solutions (PBS). All other chemicals were of analytical grade and used without further purifications.

2.2 Apparatus

CHI 1205A and CHI 900 electrochemical workstations (CH Instruments) were employed for the cyclic voltammetry (CV) and differential pulse voltammetry studies (DPV). Hitachi S-3000 H, scanning electron microscope (SEM) was used for the surface morphological investigations of the modified GCE. A conventional three-electrode system consisting of Tyr modified glassy carbon electrode(GCE) as a working electrode (active surface area =0.079 cm^2), a Ag/AgCl electrode (Sat. KCl) as a reference electrode and a platinum wire with 0.5 mm diameter as a counter electrode was employed for electrochemical experiments. All the electrochemical measurements were carried out under nitrogen saturated conditions and purged the nitrogen gas about 5 minutes before the start of each experiment.

2.3 Procedure of sample preparation

Lab sample

For lab sample preparation, 0.01M of HDH, ISN, EBH and PZM were prepared individually in to a separate 20 ml volumetric flasks in pH 7.0 PBS and subsequently sonicated for 15 minutes in cold water bath. The as-prepared sample solution was stored in the refrigerator at 4°C when not in use. The samples were prepared 30 minutes before the analysis.

Real samples (Human Blood Serum and Urine)

About 6 ml of fresh human blood was collected from a healthy person aged around 25 years and the aliquot of the sample was collected in a test tube. The collected blood sample was kept at room temperature for 30 minutes and then centrifuged for 15 minutes at 1300 rpm. Finally, the supernatant was collected in a new test tube and stored at 4°C in refrigerator when not in use. 1 ml of the supernatant was further diluted 10 times with pH 7.0 PBS to reduce the complex interferences. Aliquot of sample was prepared before 45 minutes prior to analysis. The blood serum was attentively transferred into the 25 ml electrochemical cell and analyzed without any further pretreatment.

Human urine samples were obtained from a healthy person aged around 25 years and the samples were stored in a refrigerator at 4°C. About 25 ml of urine sample was centrifuged for 15 min at 3000 rpm. 1 ml of the supernatant was collected and further 10 times diluted with pH 7.0 PBS to avoid the complex interferences. The resulting solution was attentively transferred into a 25 ml electrochemical cell and analyzed without any further pretreatment.

This research study was reviewed and approved by the ethics committee of Chang Gung Memorial Hospital, Taiwan, through the contract no.IRB99-3223C. The analysis was performed under the guidelines of the committee. Written informed consent was obtained from participants before this work.

Pharmaceutical sample (Tablets)

Isoniazid (75mg), Pyrazinamide (400mg), Hydralazine HCl (25mg) and Ethambutol HCl (100mg) tablets were procured from the medical store, further finely crushed with mortar and pestle. The powdered individual samples were exactly weighed to equalize the 0.01M concentration of ISN, PZM, HDH and EBH and carefully transferred to 25 ml volumetric flask and further dissolved with 0.05 M PBS and subsequently ultra-sonicated for 20 minutes in cold water bath. All the samples were prepared (crushed) before 30 minutes prior to analysis and were stored in refrigerator at 4°C in a closed container when not in use.

3. Results and discussion

3.1. Fabrication of Tyr-GCE and Morphological studies

Prior to modification, the GCE surface was first polished with alumina slurry on BAS polishing pad, followed by successive sonication in ethanol and double distilled water. After bath sonication, the electrode was rinsed with doubly distilled water and allowed to dry at room temperature for few minutes and employed for the electro polymerization process of Tyr. **Fig.1(A)** depicts the continuous cyclic voltammograms of electrochemical polymerization of DL-Tyrosine (Tyr) on the surface of GCE. For the electrode fabrication, we employed cyclic voltammetric technique and was fabricated by electrochemical polymerization of DL-Tyrosine on GCE surface in pH 7.0 PBS with 1 mM concentration of DL-Tyrosine, the potential was -0.8 to 1.8 V applied at a scan rate of 100 mV s^{-1}for16 cycles [38]. According to the previous reported literatures suggesting that, the polymerization of amino acids on GCE was very difficult at less than 1.5V and -0.6 V applied potentials, due to less than +1.6 potential scan rate the α-amino free radical are not formed and the polymerization of amino acids were difficult[40], thus we chose for electro polymerization of Tyr on GCE at -0.8 to 1.8 V potential range.

[Fig.1]

From the **Fig.1 (A)** clearly shows that well anodic peak was observed at 0.80V which corresponds the oxidation of Tyr monomer, further at -0.50 V the peaks increasing continuously while sweep segments proceeding, which clearly indicating that the polymerization of Tyr was taking place on GCE. After completion of 16 cycles an electro polymerized GCE was removed and dried at room temperature for few minutes, the modified GCE named as Tyr-GCE and carried out further electrochemical investigations.

Surface morphological studies were investigated for Tyr-GCE and bare GCE, **Fig. 1(B)** shows the clear smooth surface of bare GCE, further **Fig.1 (C)** depicts the electro polymerization of Tyr-GCE which clearly revealing the polymerized Tyr deposited on GCE and **Fig.1 (D)** shows the higher magnification of Tyr-GCE. From these results it is clear evident that Poly-Tyr formed a polymeric layer on GCE and provides more surface area and plays a vital role in the electrocatalytic activity in the detection of anti-tuberculosis and anti-hypertensive drugs.

3.2. Effect of scan rate

The effect of scan rate on the peak current has been studied for the HDH, ISN, EBH and PZM at Tyr-GCE in deoxygenated PBS pH7. The main purpose of this study is, in order to investigate the nature of the electrode process taking place on the modified electrode. Cyclic voltammograms were performed and recorded in the presence of 100 µM HDH, ISN, EBH and PZM individually from 20 to 500 mV s^{-1} in pH 7.0 PBS. The oxidation peak current of ISN

versus scan rate was linear when compare to its peak current plots against square root of the scan rate ($\upsilon^{1/2}$). The peak potentials also very slightly shifting towards positive side, further confirming it is an adsorption process and there is no other peak observed in the reverse scan of whole cycle, hence it is an irreversible electrochemical process. Further the corresponding equation for square root of scan rate versus peak current and a linear relationship of log I_{pa} and log υ can be expressed by following expression[41], the calibration plot was shown in **Fig.2A**.

[Fig.2]

$$I_{pa} = 0.046\ (\upsilon^{1/2} / mVs^{-1}) + 0.643,\ r = 0.996 \quad (1)$$

$$\text{Log } I_{pa} = 0.212 \log \upsilon - 0.366;\ r = 0.988 \quad (2)$$

From the above results it is clear evident that the oxidation of ISN on the surface electrode was an adsorption process. For diffusion controlled electrochemical process a linear relationship was observed between the plot of square root of scan rate ($\upsilon^{1/2}$) versus peak current[42, 43]. The oxidation of HDH at Tyr-GCE surface, the peak current was increasing continuously with scan rate and the peak potential was shifting towards more positive side with respect to scan rate. Further, the plot of square root of scan rate ($\upsilon^{1/2}$) versus peak current (**Fig. 2B**) was expressed by the following expression:

$$I_{pa} = 0.365\ (\upsilon^{1/2} / mVs^{-1}) - 0.917,\ r = 0.991 \quad (3)$$

And the linear relationship was observed between log I_{pa} and log υ, and the corresponding equation can be expressed by following expression:

$$\text{Log } I_{pa} = 0.605 \log \upsilon - 0.784;\ r = 0.990 \quad (4)$$

The obtained value of 0.605 is very close to the theoretical value of 0.500, which clearly indicates a diffusion controlled electrode process [42, 43]. Shifting of peak potential was observed while increasing the scan rates of EBH and peak current increases linearly with increasing the square root of scan rate at Tyr-GCE surface. The plot of square root of scan rate ($\upsilon^{1/2}$) versus peak current and a relationship of log I_{pa} versus log υ (**Fig.2C**) was expressed by the following expressions:

$$I_{pa} = 0.058\ (\upsilon^{1/2} / mVs^{-1}) + 0.453,\ r = 0.995 \quad (5)$$

$$\text{Log } I_{pa} = 0.346 \log \upsilon - 0.691;\ r = 0.998 \quad (6)$$

Similarly for PZM, upon increasing the scan rates the I_{pa}/I_{pc} ratio increases and the reaction was reversible process[21], further the cathodic peak of PZM slightly shifted towards the more negative direction. The plot of square root

of scan rate ($\upsilon^{1/2}$) versus cathodic peak current and a relationship of log I_{pc} versus log υ (**Fig.2D)** was expressed by the following expressions:

$$I_{pc} = 0.172\ \upsilon^{1/2} + 0.059,\ r = 0.985 \quad (7)$$

$$\text{Log } I_{pc} = 0.462 \log \upsilon - 0.663;\ r = 0.992 \quad (8)$$

In addition, the log υ values of PZM and EBH were of 0.462, 0.346 respectively and these were very close to the theoretical value of 0.500 hence the reduction of PZM and oxidation of EBH at Tyr-GCE could be a diffusion controlled electrochemical process. Further a calibration plot of relationship between log *I* and log υ clearly shown in the **Fig.S1**.

3.3. Influence of pH

The Influence of pH at Tyr-GCE towards electrocatalytic activity of HDH, ISN, EBH and PZM have been evaluated individually in various buffer solutions (pH 3 to 10) containing each 250 µM concentration and for EBH we studied from pH 7 to 10 with 250 µM concentration. The obtained plots of peak potentials versus different pH were shown in the **Fig.3 (A) HDH, (B) ISN, (C) EBH and (D) PZM**. The oxidation peaks of ISN and HDH showed maximum catalytic performance in pH 7 PBS, in addition the anodic peak of EBH showed good response at pH 8.0 and for PZM the reduction peak was observed good enhancement at pH 7.0 PBS than other pH buffers solutions. Further the anodic peak potentials (E_{pa}) of ISN, HDH and EBH shifted to lower potentials with the increase in pH (figure not shown.).According to the Nernstian equation for equal number of proton and electron transfer process is $\Delta E/\Delta$ pH = (59.1 mV/n) * N^{+}_{H} (n=2) and the obtained slope values for all the four drug analytes were 56, 55, 29 and 75 mV pH^{-1} respectively for HDH, ISN, EBH and PZM. The HDH and ISN slope values were very close to the Nernstian slope value of 59.1 mV pH^{-1}. Therefore, based on the above results, for ISN and HDH the number of protons (N^{+}_{H}) and electrons transfer could be two, and the linear dependence of peak potential on different pH solutions can be expressed by the following expression:

$$HDH:\ E_P / \text{V (vs. Ag/AgCl)} = -0.056\ (\text{pH}) + 0.616;\ R^2 = 0.985 \quad (9)$$

$$ISN:\ E_P / \text{V (vs. Ag/AgCl)} = -0.055\ (\text{pH}) + 0.808;\ R^2 = 0.964 \quad (10)$$

$$EBH:\ E_P / \text{V (vs. Ag/AgCl)} = 0.029\ (\text{pH}) + 0.886;\ R^2 = 0.973 \quad (11)$$

PZM: E_P / V (vs. Ag/AgCl) = -0.075 (pH) - 0.290; R^2= 0.970 (12)

[Fig.3]

In addition the ISN reaction mechanism was shown in the **Scheme-1a**[44, 45] and the HDH mechanism was unclear. Similarly for the PZM, slope value is -75.0 mV pH^{-1}, hence the number of protons (N^+_H) transfer could be three, the above results were complying with the electrochemical behavior of pyrazine in the mercury electrode[46, 47].In addition, the redox reaction of the PZM leads to formation of 1,4-hidropiraziniumion the mechanism was shown in the **Scheme.1b**[21]. Finally for the EBH, the obtained slope value is 29 mV pH^{-1}, which is half of the Nernstian slope value 59.1 mV pH^{-1}, and proton is directly involving,[10] hence it could be one proton transfer. Further we consider for physiological and analytical perspective, we selected the pH 7 (PBS) solution for proceed all of our further analysis.

[Scheme.1]

3.4. The electrochemical behaviors of HDH, ISN, EBH and PZM at Tyr-GCE

The electrochemical behaviors of HDH, ISN, EBH and PZM at Tyr-GCE and bare GCE were investigated using CV. **Fig.4** shows the cyclic voltammograms of 150 µM of HDH, ISN, EBH and PZM in 0.05M pH 7 PBS with a scan rate of 100 mV $s^{-1}$for Tyr-modified GCE (a) and bare GCE (a) under nitrogen saturated condition (5 minutes purged). On the bare GCE the oxidation of HDH, ISN were observed at 0.18 and 0.65 V respectively, for EBH there is no response was observed, in addition a sharp cathodic peak was observed at -1.14V for PZM. On other hand for Tyr-GCE, the electrocatalytic oxidation of HDH, ISN and EBH were occurred at a potential of 0.14, 0.55, 1.12 respectively, similarly for PZM the reduction was observed at a potential of -0.85V. These results were clearly shows that the peak potentials were shifted towards less positive side at modified GCE than bare GCE, further I_{pa} of HDH and ISN were almost six fold times more than bare GCE. Similarly I_{pc} of PZM at Tyr-modified GCE was two folds more than bare GCE due to the polymeric film is more stable with more real surface area, possessing of amino, carboxylic active sites in its structure and further enhancing the conductance at the surface of GCE.[38]

[Fig.4]

3.5. Electrochemical detection of HDH, ISN, EBH and PZM at Tyr-GCE by Differential Pulse Voltammetry

DPV technique was employed to obtain the linear ranges and detection limits for the HDH, ISN, EBH and PZM drugs. The DPV voltammograms were recorded at a constant time interval of 90 seconds with nitrogen gas

purging before the start of each experiment. The optimization of DPV method was discussed in S2 (Supporting information file).We have employed the following parameters of 50 mV for pulse amplitude, 0.05 ms for pulse width and 200 ms for pulse period for all the DPV analysis. And the DPV is a good sensitive and high resolution technique compared to CV [48]. **Fig. 5A** displays the DPV response of an electrochemical oxidation of HDH at Tyr-GCE in pH7.0 PBS under the optimized working conditions at 0.17 V. The anodic peak current was increased linearly with continuous additions of HDH and a linear relationship was established in the range of 6 to 180 μM (**Inset of Fig. 5A**) and the sensitivity was calculated from the fitted regression equation as 0.228 μA μM^{-1} cm^{-2}.

[Fig.5]

Similarly **Fig.5B** and **Fig.5C** depicts the DPV responses for electrochemical oxidations of ISN, EBH respectively. The anodic peaks of both ISN, EBH were linearly increased with continuous additions of its concentrations in the electrochemical cell. A linear range of 25 to 1250 μM and 20 to 1000 μM concentrations were obtained for ISN and EBH respectively. Further the sensitivity was calculated for ISN as 0.06 and EBH as 0.09 μA μM^{-1} cm^{-2} respectively.

From the **Fig.5D** it can be seen that, the electrocatalytic reduction peak current of PZM at Tyr-GCE was linearly dependent on PZM concentration. PZM reduction peak was increased while continuous additions of PZM content in to the electrochemical cell, further a wide linearity was obtained from 10 to 900 μM, and the sensitivity was calculated from the fitted regression equation as 0.165 μA μM^{-1} cm^{-2}. The superior performance of the Tyr modified GCE showed a promising platform for the electrochemical determination of HDH, ISN, EBH and PZM.

3.6. Stability, repeatability and reproducibility

In order to examine the film stability of modified electrode at room temperature, it was stored in dry place and the back ground current was monitored for three weeks by CV technique in the pH 7.0 PBS. The modified electrode retained about 95.1% of its initial current, representing the modified electrode has good film stability. Further, the background current was 94.2% stable even after 150 consecutive cycles performed by CV technique in pH 7.0 PBS and these obtained results clearly indicating, that the proposed film has excellent film stability. The repeatability and reproducibility of the proposed sensor were evaluated by DPV studies. For reproducibility, we employed separately five Tyr-GC electrodes. Further this showed an acceptable reproducibility with a relative standard deviation (RSD) of 2.91, 2.82, 2.76 and 2.40 % respectively for HDH, ISN, EBH and PZM in 150 μM of each analytes by DPV technique.

Moreover the modified GCE showed an excellent repeatability with RSD value of about 2.22, 2.48, 2.85 and 2.71 % for HDH, ISN, EBH and PZM respectively by DPV technique with 10 successive measurements in the presence of 150 µM concentration of each analyte, the obtained values suggesting that the modified electrode has good repeatability and reproducibility.

3.7 Interference study

In order to investigate the possible interfering electroactive species for the proposed method, we monitor the response of proposed electrode by cyclic voltammetry technique and the electrochemical method conditions are similar to Sec.3.4. Further 150 µM of HDH, ISN, EBH and PZM in the pH 7.0 PBS were added separately and 40 µM of sucrose, starch, lactose, addition magnesium stearate, Na+, Ca2+, K+, are added in to the each above mentioned analytes in an electrochemical cell. The addition of each electroactive interfering species shows hardly discernible current response, whereas notable response is observed for PZM for lactose addition but it does not affect any changes in the peak current for PZM. The results clearly show that the presence of these substances do not affect the peak potential of HDH, ISN, EBH and PZM analytes. Hence these results validates that the electro active species does not affect the response currents of above mentioned drugs and they are quite negligible. It suggests that Tyr-GCE film is well selective for the detection of HDH, ISN, EBH and PZM.

3.8. Method validation for the proposed method

According to the International Conference on Harmonization (ICH) guidelines[1-3] the limit of detection (LOD), limit of quantitation (LOQ) and specificity were calculated.

Detection limit (LOD)

For the LOD calculation, first need to calculate the slope value (S) from the calibration curve of linearity. The linearity was achieved by the response of obtained current versus theoretical concentrations of for HDH, ISN, EBH and PZM were plotted and calculated the linear regression equation, standard deviation (σ) of response was calculated for the blank responses (Signal-to-noise ratio for 3 runs S/N=3) . The LOD was calculated according to the following ICH guidelines equation

The detection limit **(LOD) =3.3 σ/S**

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The obtained LOD values for the HDH, ISN, EBH and PZM were 5.66, 6.93, 9.61 and 5.13 µM respectively. Further the obtained LOD values were good agreement with the method, found less than the initial linearity values and these results showed that the method is within the desired range.

*Quantification limit (**LOQ**)*

The LOQ was calculated, according to the ICH guidelines by the following equation

Quantification limit **(LOQ) =10 σ/S**

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The obtained LOQ values were calculated for HDH, ISN, EBH and PZM as found 17.18, 21.05, 29.15 and 15.556 µM respectively.

Specificity

Specificity is the ability of the method to assess the analytical response of the analytes in the presence of excipients and potential impurities. For the specificity test, voltammograms of the standard solutions of tablet excipients (starch, sucrose, lactose and magnesium stearate) were recorded. The response of the HDH, ISN, EBH and PZM analytes in this mixture was compared with the response of pure HDH, ISN, EBH and PZM. It was found that assay results were not changed, further no interference was observed from the existing excipients and the proposed method has good specificity for the above mentioned analytes.

3.9 Analytical applications

Determination of HDH, ISN, EBH and PZM *in Biological samples (Human Urine and Blood serum)*

For the practicality of proposed method, we investigated the concentration level of each analyte in biological samples by DPV technique. The prepared urine sample preparation stated in the section 2.3 (Real sample preparation) was spiked with a known concentration of individual analytes. The concentrations of each analyte in the real sample

urine and the spiked samples were determined by using the standard addition method which showed good recovery results and the results were summarized in the **Table.1** and the calculated relative standard deviation (RSD) was less than 4%.The obtained results indicate that the proposed sensor can be effectively used for the real sample analysis to detect HDH, ISN, EBH and PZM without any complex interference present in the urine samples. Similarly, we have demonstrated further in blood serum and the sample preparations were mentioned in the section 2.3 (Real sample preparation). Standard addition method was employed to calculate recoveries, and the obtained results were summarized in the **Table. 1**. These results were clearly indicating that the detection procedures were free from biological interferences of the blood serum sample matrix, further it is obvious that the current method is more suitable and could be utilized to detect the above mentioned drugs in clinical samples.

[Table.1]

Pharmaceutical analysis of HDH, ISN, EBH and PZM

In order to verify the applicability and reliability of the proposed sensor, the DPV technique was employed for the determination of HDH, ISN, EBH and PZM in commercially available pharmaceutical tablets. The sample preparation procedures are clearly mentioned in stated in the section 2.3 (Pharmaceutical sample). For the working standard here we used lab sample and the electrolyte is 0.05M of PBS (pH 7). The concentration of HDH, ISN, EBH and PZM in the pharmaceutical formulations was determined by assay method. Further the quantified sample values were very close to the claimed amount of the tablets and the analytical performances were summarized in **Table.2**. The obtained results were in good agreement with the claimed label amount of the tablets and the RSD (n=3) is less than 4%. The obtained results clearly show that the existing drug excipients in the commercially available tablets do not have any significant interference. Thus the proposed method is promising for the direct determination of HDH, ISN, EBH and PZM in pharmaceutical samples.

[Table.2]

4. Conclusions

In this current research work, the proposed Tyr-GCE exhibits high electrocatalytic activity for the PZM, HDH, ISN and EBH. The proposed modified electrode further exhibits good sensitivity, film stability, high reproducibility and

repeatability. In addition practicality of the proposed sensor was successfully demonstrated in biological samples (blood serum and urine). The applicability of the proposed sensor was confirmed with pharmaceutical formulation tablets, the obtained results were good agreement with the label claim of the tablets. The proposed method has low detection limit and wide linear range for the above mentioned four analytes. Moreover, the proposed electrode is easy to fabricate, low cost and more reliable, further the proposed method could be utilized for routine analysis in clinical and pharmaceutical samples in near future.

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References:

1 S. N. M. Hanif, A. J. Hickey and L.G. Contreras, *J. Pharm. Biomed. Anal.,* 2014, **88**, 370-376.

2 M. D. J. A. Khan and Z. Ahmed, *International Journal of General Medicine and Pharmacy.*, 2013, 2**,** 2319-2399.

3 P. Nagaraja, K. C. S. Murthy and H.S. Yathirajan, *Talanta*., 1996, **43,** 1075-1080.

4 M. Y. Khuhawar and F. M. A. Rind*, J. Chromatogr. B*., 2002, **766**, 357-363.

5 M. Moser and P. R. Hebert, *J Am CollCardiol*., 1996, **27,** 1214-1218.

6 B. Singh, S. Pahuja, R. Kapil and N. Ahuja, *Acta Pharm*., 2009, 59, 1-13.

7 R. V. Hest, H. Baars, S. Kik, P. V. Gerven, M. C. Trompenaars, N. Kalisvaart, S. Keizer, M. Borgdorff, M. Mensen and F. Cobelens, *Clin Infect Dis*., 2004, **39,** 488-496.

8 S. Tafazoli, M. Mashregiand P. J. O'Brien, Toxicol. *Appl. Pharmacol.,*2008, **229,** 94-101.

9 C. I. Zoumalan, M. Agarwal, A. A. Sadun and Graefe's Arch. *Clin. Exp. Ophthalmol*., 2005, **243**, 410-416

10 C. Shao, W. Yingliang, L. Shumianand L. Chao, *Chem. Anal.,* 2008, **53,** 511-521.

11 C. K. Ma, C. S. Mok and K. H. Ping, *Anal. Chim. Acta*., 1995, **314**, 77-85.

12 S. Jin, S. T. Hong and Z. T. Hong, *Chin. J. Chromatogr.,*2006, **24,** 164-167.

13 M. R. Holdiness, Z. H. Israili and B. Joseph, *J Chromatogr B Biomed Sci Appl.,* 1981, **224**, 415-421.

14 Y. C. Hsieh and H. W. Wang, *J. Chromatogr. A.,*2006, **1122**, 279-282.

15 S. M. Saad and H. A. Shalaby, *Microchim. Acta., 1992,* **109,** 193-199.

16 P. R. D. Oliveira, M. M. Oliveira, A. J. G. Zarbin, L. H. MarcolinoJr and M. F. Bergamini, *Sens. Actuators, B.,* 012, **171-172,** 795-802.

17 A. Safavi, M. A. Karimi, M. R. H. Nezhad, R. Kamali and N. Saghir, *Spectrochim. Acta,* Part A., 2004, **60**, 765-769.

18 B. Wu, W. Wang, Z. Xue, X. Zhou, J. Du, X. Liu X and X. Lu, *Analyst.,* 2012, **137**, 3644-3652.

19 S. Cheemalapati, S. Palanisamy and S. M. Chen, *Int. J. Electrochem. Sci. ,*2013, **8,** 3953-3962.

20 A. F. Faria, M. V. N. Souza, R.E. BrunsandM.A.L. Oliveira,*Talanta.*, 2010, **82,** 333-339.

21 M. F. Bergamini, D. P. Santos and M. V. B. Zanoni, *J. Electroanal. Chem.,* 2013, **690,** 47-52.

22 S. Cheemalapati, B. Devadas and S.M. Chen, *J. Colloid Interface Sci.,*2014, **418,** 132-139.

23 A. K. Chaitanya, R. S. Saravanan, S. Jeevanantham, R. Vignesh and P. Karthik, *Adv. Pharmacoepidem. Drug Saf.,* 2012, **1**, 1-5.

24 J. Madan, A. K. Dwivedi and S. Singh, *Anal. Chim. Acta.,* 2005, **538**, 345-353.

25 Z. Gong, Y. Basir, D. Chu and M.M. Tipton, *J. Chromatogr. B.,*2009, **877,** 1698-1704.

26 H. M. Maher and R. M. Youssef, *Chemom. Intell. Lab. Syst.,*2008, **94,** 95-103.

27 S. Cheemalapati, S. Palanisamy, and S. M. Chen, Int. *J. Electrochem. Sci.,* 2012,**7,**11488-11496.

28 Y. Xiong, H. Zhou, Z. Zhang, D. He and C. He*, J. Pharm. Biomed. Anal.,* 2006, **41**, 694-700.

29 K. D. Haegele, H. B. Skrdlant, N. W. Robie, D. Lalka and J. L. McNay, *J. Chromatogr.,*1976, **126,** 517-534.

30 P. H. Degen, *J. Chromatogr.,*1979, **176,** 375-380.

31 J .K. Wong, T. H. Joyce III and D.H. Morrow, *J. Chromatogr.,* 1987, **385,** 261-266.

32 J. T. Stewart and E. H. Parks*, Int. J. Pharm.,*1983, **17,** 161-166.

33 H. R. Angelo, J. M. C. Kristensen and A. McNair, *J. Chromatogr.,*1980, **183,** 159-166.

34 A. M. D. L. Pena, F. Salinas and M.S. Duran, *Anal. Chim. Acta.,*1991, **255,** 317-323.

35 W. Gao, J. Song and N. Wu, *J ElectroanalChem.,* 2005, **576**, 1-7

36 R. Thangamuthu, Y. C. Pan and S.M. Chen*, Electroanal.,*2010, **22,** 1812-1816.

37 M. Wei and S.D. Ming, *Chin. J. Anal. Chem.,* 2007, **35,** 66-70.

38 Y. Wang and B. Chunyan, *J. Mol. Liq.,*2013, **177,** 26-31.

39 L. Fotouhi and R. Tabatabaee, *Spectrochim. Acta,* Part A., 2014, **121**, 152-156.

40 C. Jiang, T. Yang, K. Jiao and H. Gao*, Electrochim. Acta.,* 2008, **53,** 2917-2924.

41 A. J. Bard and L. R. Faulkner*, Electrochemical Methods: Fundamentals and Applications*,2ndedn, Wiley, New York, 2000.

42 D. K. Gosser, *Cyclic Voltammetry: Simulation and Analysis of Reaction Mechanisms*, Wiley−VCH Publishers, New York, 1993, pp 27-45.

43 B. K. Chethana, S. Basavanna and Y. A. Naik, *Ind. Eng. Chem. Res.,* 201, **51**, 10287-10295.

44 K. Johnsson and P. G. Schultz,*J. Am. Chem. Soc.,*1994, **116,** 7425-7426.

45 P. A. Uday and G. Vellaichamy, *J. Solid State Electrochem*.,2012, **16,** 2907-2911.

46 Y. Ni, S. Kokot, M. Selby andM. Hodgkinson*, Electroanalysis.,* 1992, **4,**713-718.

47 F.C. Anson, J. M. SaveantandK. Shigeharat, *J. Am. Chem. Soc.,*1983, **105,**1096-1106.

48 A. A. Ensafi, M. TaeiandT. Khayamian, *J. Electroanal. Chem.,* 2009, **633,** 212-220.

49 Note for guidance on validation of analytical procedures: methodology, (CPMP/ICH/281/95) ICH topic Q2B, ICH Technical Coordination, London, UK, 1996

50 Note for guidance on validation of analytical procedures: text and methodology, (CPMP/ICH/381/95) ICH topic Q2 R1, ICH Technical Coordination, London, UK, 1996

51 M. Swartz and I.S. Krull, Analytical method development and validation. Marcel Dekker, 1997, p 62

Table.1 Determination of HDH, ISN, EBH and PZM in Human urine and blood serum samples

Sample labeled	Analyte	Added (μM)	Found (μM)	Recovery (%)	RSD[a] (%)
Urine sample	HDH	100	99	99	2.1
Urine sample	ISN	100	98	98	2.5
Urine sample	EBH	100	97	97	3.1
Urine sample	PZM	100	99.5	99.5	2.1
Blood Serum	HDH	90	88.5	98.3	2.8
Blood Serum	ISN	90	87.5	97.2	3.1
Blood Serum	EBH	90	87	96.6	3.4
Blood Serum	PZM	90	89	98.8	2.7

[a] Relative Standard Deviation

Table.2 Determination of HDH, EBH, ISN and PZM in pharmaceutical formulation tablets

Sample labeled	Content(mg[a])	Added (mg)	Found[b] (mg)	Recovery (%)	RSD[c] (%)
HDH Tablet 1	25	-	24.6	98.4	3.1
HDH Tablet 2	25	10	34.3	98	3.5
EBH Tablet 1	100	-	99.1	99.1	2.5
EBH Tablet 2	100	10	108	98.2	3.6
ISN Tablet 1	75	-	74.2	98.9	3.2
ISN Tablet 2	75	10	83.9	98.7	3.3
PZM Tablet 1	400	-	401.1	100.3	2.4
PZM Tablet 2	400	10	411.1	100.3	2.3

[a] Milli gram

[b] Average of three determinations

[c] Relative Standard Deviation

Figure captions

Fig.1 A shows CVs of 1mM concentration of DL-Tyr (Supporting electrolyte is PBS pH 7.0) electrochemical polymerization on GCE (16 cycles) with a scan rate of 100 mVs^{-1}, in -0.8 to 1.8V potential range. **Fig.1B** shows the SEM image of bare GCE, **C** shows the lower magnification of Tyr-GCE and **Fg.1D** is the higher magnification of Tyr-GCE

Fig.2 Relationship between anodic peak current and square root of scan rate of 100 μM ISN (**A**), HDH (**B**), EBH (**C**) and relationship between cathodic peak current and square root of scan rate of 100 μM PZM (**D**) from 20 to 500 mV s^{-1} in pH 7.0 PBS at Tyr-GCE

Fig.3 Relationship between peak potential and solution pH at Tyr-GCE in the presence of 250 μM concentration of each HDH (**A**), ISN (**B**), EBH (**C**) and PZM (**D**) drugs recorded individually by CV techniques, the pH ranges are 3- 10 at the scan rate of 100 mV s^{-1}.

Fig.4 CVs of 150 μM of each HDH, ISN, EBH and PZM at bare (**a**) and Tyr modified GCEs (**b**), Conditions: scan rate 100 mVs^{-1}, deoxygenated PBS pH 7.0

Fig. 5A DPVs of Tyr-GCE response for the different concentrations of HDH in pH 7 PBS [curve a- without addition of HDH (only in the electrolyte solution), HDH concentrations were in the range of 6-180 μM (b-k), Inset is the linear dependence of I_{pa} vs. concentration of HDH]. **Fig. B** depicts the DPV response of ISN in PBS [a- without addition of ISN (only in the electrolyte solution), ISN in the range of 25-1250 μM (b-k)], Inset is the linear dependence of I_{pa} vs. concentration of [ISN]. **Fig. C** depicts the DPV response of EBH in PBS [In the range of 20-1000 μM (b-k), a- without addition of EBH (only in the electrolyte solution), Inset is the linear dependence of I_{pa} vs. concentration of EBH] and **Fig. D** depicts the DPV response of PZM in PBS [In the range of 10-900 μM (b-o), a- without addition of PZM (only in the electrolyte solution), Inset is the linear dependence of I_{pc} vs. concentration of PZM].

Scheme. 1 Formation of Isonicotinic acid mechanism (**a**) and 1,4-hidropiraziniumion formation mechanism (**b**)

Figures:

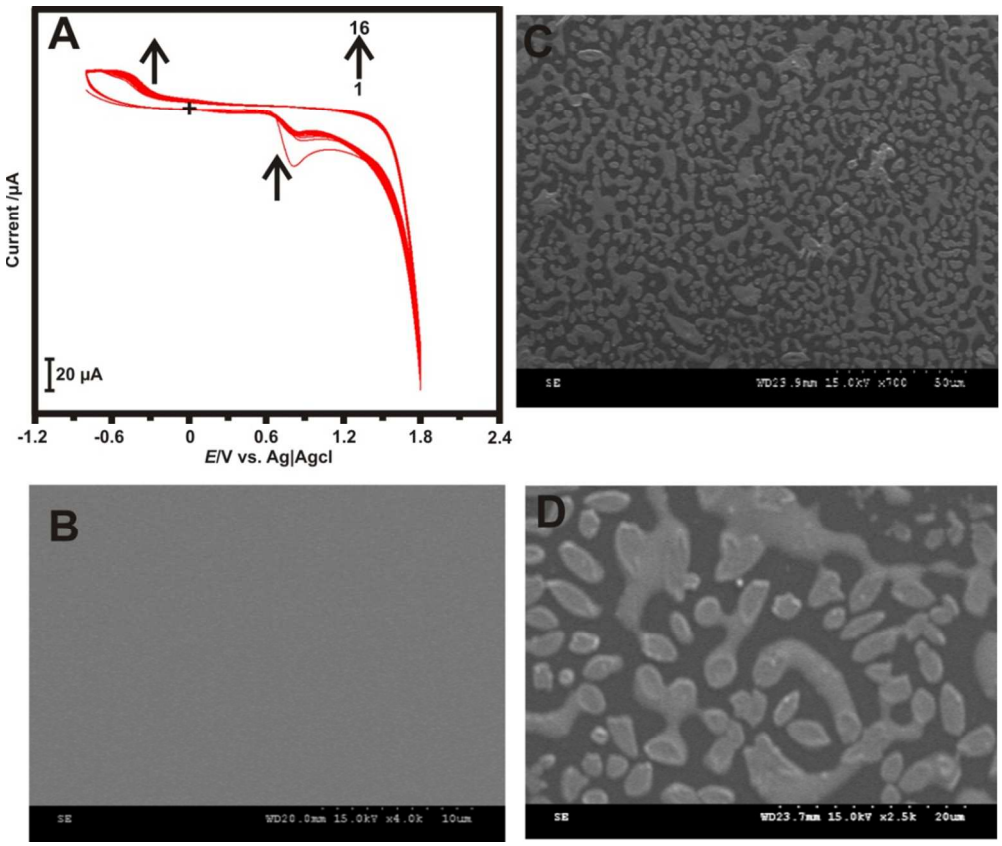


Fig.1

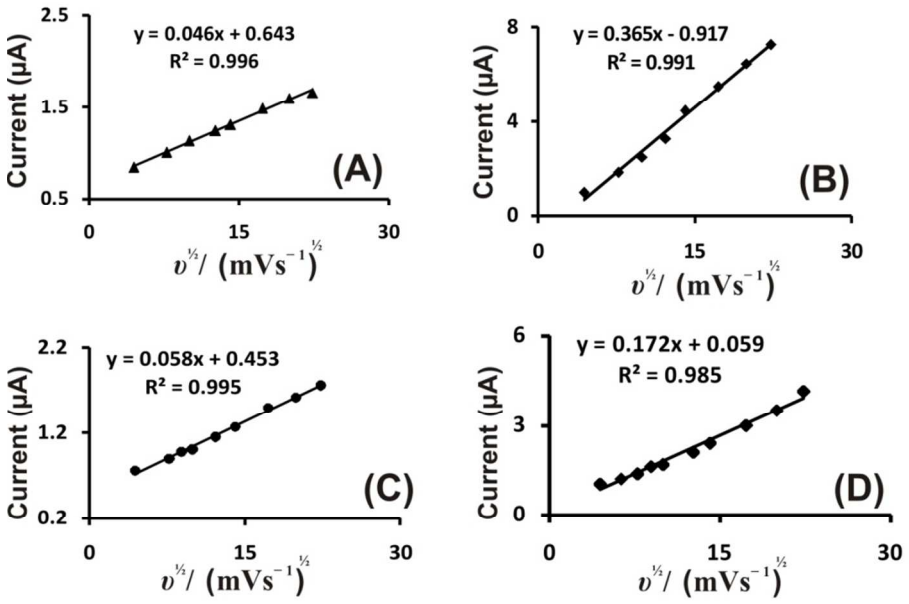


Fig.2

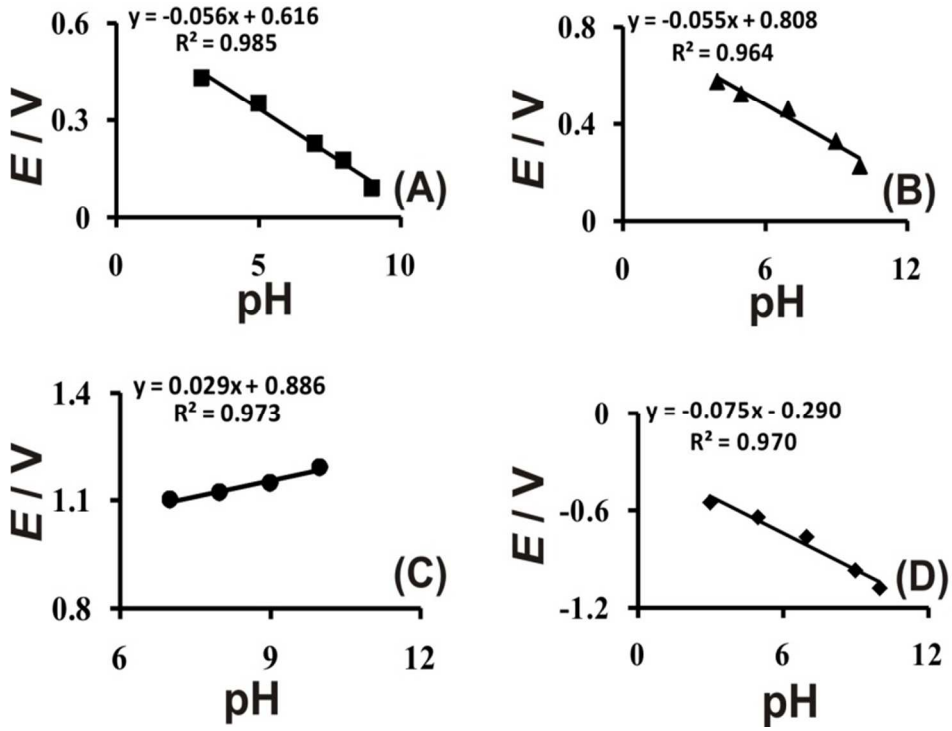


Fig.3

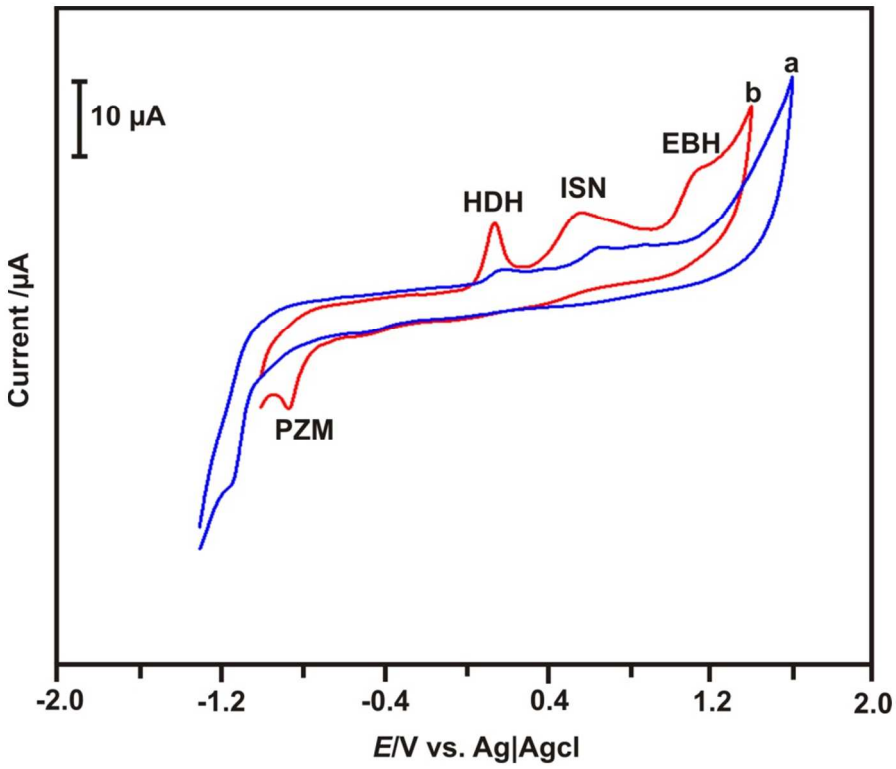


Fig.4

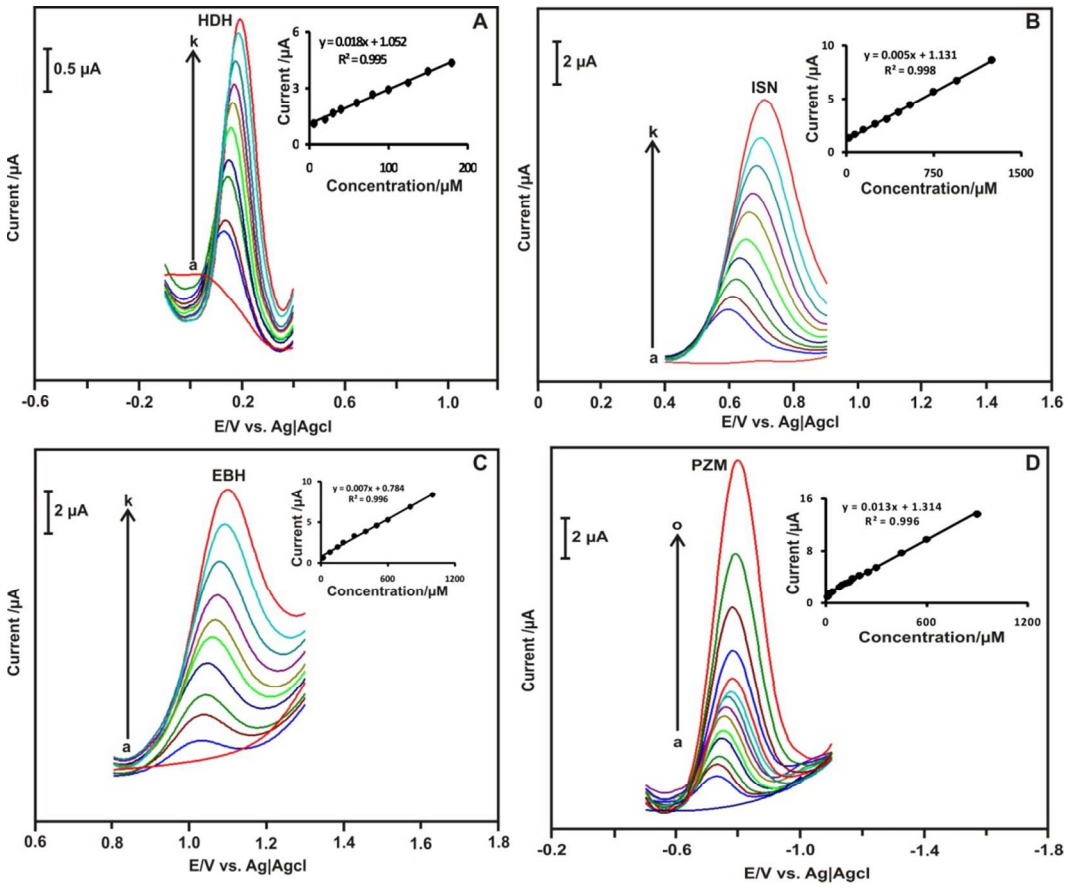
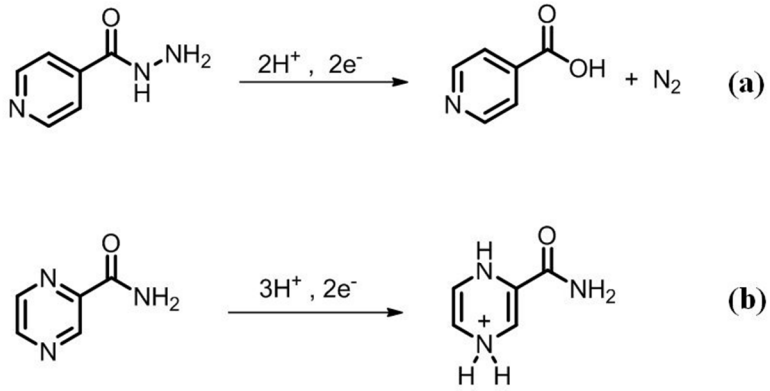


Fig.5



Scheme.1