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Reduction Pathways of Zofenopril Based on Experimental and Computational Approach and its Voltammetric Determination

İbrahim Hüdai Taşdemir[1,*] *and Esma Kılıç*[2]

[1]Amasya University, Faculty of Arts and Science, Department of Chemistry, 05200 Amasya/TURKEY
[2]Ankara University, Faculty of Science, Department of Chemistry, 06800 Ankara/TURKEY
[*]E-mail: ibrahim.tasdemir@amasya.edu.tr, ibrahimhudaitasdemir@gmail.com

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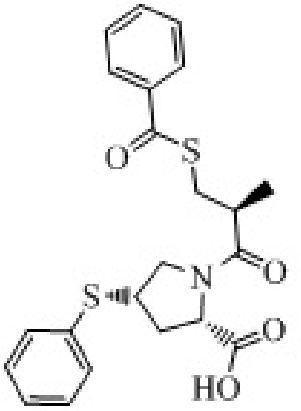
Electrochemical reduction behavior of Zofenopril (ZFN) was studied via experimental electrochemical methods and theoretical calculations performed at B3LYP/6-31+G (d)//AM1. Electrochemical studies based on one reversible and adsorption-controlled and two irreversible diffusion-controlled reduction peaks at -0.75 V, -1.3 V and -1.65 V, respectively on hanging mercury drop electrode (HMDE) versus Ag/AgCl, KCl (3.0 M) in Britton-Robinson buffer (BR) of pH 5.5. First peak (reversible one) was thought to be the reduction of carbonyl group activated by vicinal nitrogen and reoxidation to its original form, second reduction was supposed to be the reduction of other carbonyl and reduction of proton to hydrogen gas catalyzed by lone-pair electrons was proposed as third. Square-wave cathodic adsorptive stripping voltammetry has been developed and validated for quantification of ZFN in different samples. Linear working range was established as 0.03-1.05 µM. Limit of determination (LOD) was calculated to be 0.01 µM. Proposed method was successfully applied to assay the drug in tablets and human serum with good recoveries between 96.9 % and 101.4 % having relative standard deviation less than 10 %.

Keywords: ab-initio calculations, electrochemical behavior, voltammetry, stripping analysis, zofenopril

1. INTRODUCTION

High blood pressure is still very common public health problem all over the word. Various drugs are used for its treatment in different drug category. One of the important category is angiotensin converting enzyme inhibitor and zofenopril (ZFN), chemically known as (2S,4R)-1-((S)-3-(benzoylthio)-2-methylpropanoyl)-4 (phenylthio)pyrrolidine-2-carboxylic acid (scheme 1), is one

member of this class and with its cardio protective properties it is used for treatment of high blood pressure either alone or in combination with some diuretics [1,2].



Scheme1. Chemical structure of ZFN

Since it is a drug active molecule mainly chromatography-based detection and determination methods such as LC - tandem mass spectrometry [3], HPLC with DAD detection [4], spectrophotometry [5], GC with mass detection [6], densitometric method [7], RP-LC [8], LC-ESI-MS [9] and LC–tandem mass spectrometry method [10] have been devised and reported.

Chromatography-based methods have many advantageous on detection and determination of species including drug molecules. Especially for mixture of multi-component analysis these methods are of great importance. Chromatographic methods alone are either not sensitive enough or to increase their sensitivity tedious pretreatment and highly sophisticated instrumentation are required.

Electrochemical methods might be used in investigating many physical, chemical and redox behavior of species. Such properties and their evaluation are of great importance for biologically important molecules and drug compounds. These methods also make it possible to evaluate the redox characteristics, to propose the plausible mechanism pathways, to evaluate the adsorption-diffusion parameters of molecules and these parameters may have importance for their distribution, pharmacological, toxicological and pharmacokinetic properties. Voltammetric techniques are generally used for the quantitative determination of electroactive species. Furthermore, voltammetric stripping methods extends the usage of such methods because of lower detection limits. ZFN molecule has electrochemically active groups but there is no further study carried out to deal with its electrochemical properties and its voltammetric determination without our previous results [11].

The present study was designed to investigate the cathodic behavior of ZFN on hanging mercury drop electrode (HMDE) with the support of computational studies. Tentative reaction mechanisms were also proposed. In addition, it was also aimed to develop rapid, simple and novel adsorptive stripping method for its direct determination in pharmaceutical dosage forms and human serum.

2. EXPERIMENTAL

2.1. Apparatus

Voltammetric measurements were carried out using CHI760D electrochemical analyzer (CH Instrument Inc. Austin, USA). The three electrode system consisted of working electrode (hanging mercury drop electrode (HMDE); BAS CGME 1108, 0.0145 cm^2), reference electrode (Ag/AgCl/3M KCl; MF-2052, RE-5B) and a Pt auxiliary electrode (BAS MW-1034) were used. Prior to each experiment, electrochemical cell content was degassed with argon and during measurements cell was blanked by argon.

All pH measurements were made with Thermo Orion Model 720A pH ion meter having an Orion combined glass pH electrode (912600; Thermo Fisher Scientific). Double-distilled deionized water was supplied from Ultra-Pure Water System (ELGA as PURELAB Option-S). All measurements were performed at room temperature (21±3°C).

2.2. Reagents and Solutions

Standard sample of ZFN (99.0%, from A. Menarini Industrie Farmaceutiche Riunite Srl, Florence – Italy, as calcium salt) was used and its stock solutions (5.0 mM,) were prepared in methanol and kept in the dark and below 4 °C. Working ZFN solutions were prepared by sufficient dilution of stock solution with supporting electrolyte on optimum pH and used within the day to avoid possible decomposition. Phosphoric acid (Riedel-de-Haen, Honeywell Specialty Chemicals Seelze GmbH, Germany), boric acid (Riedel-de-Haen, Honeywell Specialty Chemicals Seelze GmbH, Germany) and acetic acid (Merck KGaA, Darmstadt, Germany) were used in the preparation of BR solution in which each component had an analytical concentration of 0.04 M. Double-distilled deionized water was used in preparations of all solutions. All chemicals were used as received.

2.3. Procedure

For voltammetric measurements, a known volume of ZFN solution was pipetted into 10.0 mL supporting electrolyte. Voltammetric measurements were carried out after degassing with argon for 5 min. Voltammograms were then recorded by scanning the potential towards the negative direction versus reference electrode.

A three-electrode combination system for bulk electrolysis (BE) with mercury pool (55.4 cm^2) as working electrode, coiled platinum wire as an auxiliary electrode (BAS MW-1033 (23 cm)) and Ag/AgCl reference electrode (BAS MF-2052 RE-5B in 3.0 M KCl) was used. In BE studies 25 mL of 0.1 mM solution was used.

2.4. Preparation of Zoprotec® tablets and spiked human serum

Zoprotec® tablets achieved commercially from the pharmacy in Ankara, Turkey and were used as pharmaceutical dosage form that contains 30 mg ZFN per tablet. Preparation of tablet solutions and human serum samples were performed as described before [11].

2.5. Computation

Theoretical calculations were performed to support the proposed mechanism for electrode processes. All calculations were performed with the Gaussian 09 suite of programs [12]. The geometry of ZFN was fully optimized at AM1 level. Frequency calculations were computed at the same level to verify that the optimized geometry is a real minimum on the potential energy surface without any imaginary frequency. Single point energy calculation was done using AM1-optimized geometry at DFT/B3LYP level of theory, with the popular polarized basis set, 6-31+G (d) which adds d functions on heavy atoms.

3. RESULTS AND DISCUSSION

Cathodic electrochemical behavior, diffusion and adsorption properties of ZFN were studied on HMDE using methods such as cyclic voltammetry (CV), square-wave voltammetry (SWV) and constant potential bulk electrolysis (BE).

3.1. Electrochemical behavior of ZFN on HMDE

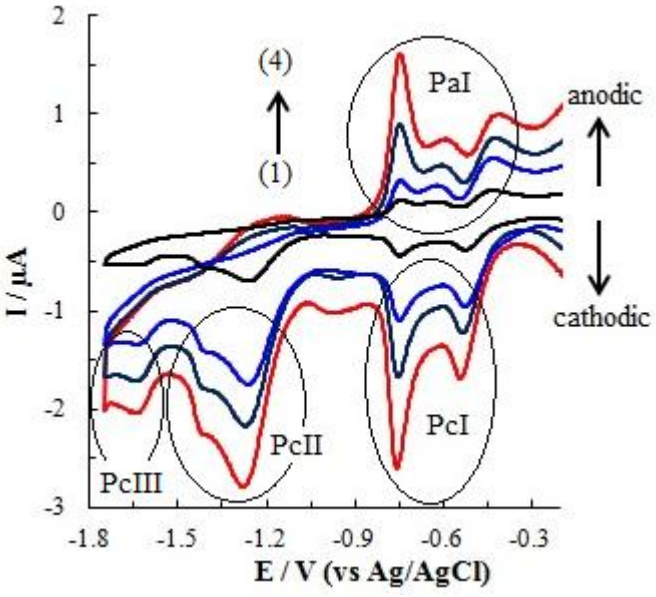


Figure 1. CVs of ZFN solutions with different concentrations (1) 0.5, (2) 1.0, (3) 1.5, (4) 2.0 mM in BR of pH 5.5, Scan rate: 0.100 Vs^{-1}

According to CV studies, one reversible reduction-oxidation wave between potentials -0.6 V and -0.8 V (labeled as PcI and PaI) and two irreversible reduction peaks at -1.3 V (labeled as PcII) and -1.65 V (labeled as PcIII) were obtained (Fig.1). As could be seen in Fig.1, current of these peaks increased with increasing ZFN concentration. Since there is oxidation peak on reverse scan for PcI, this reduction should be reversible whereas there is no oxidation couple for PcII and PcIII indicating the irreversible nature [13-16] for these reductions.

3.1.1. Effect of Potential Scan Rate

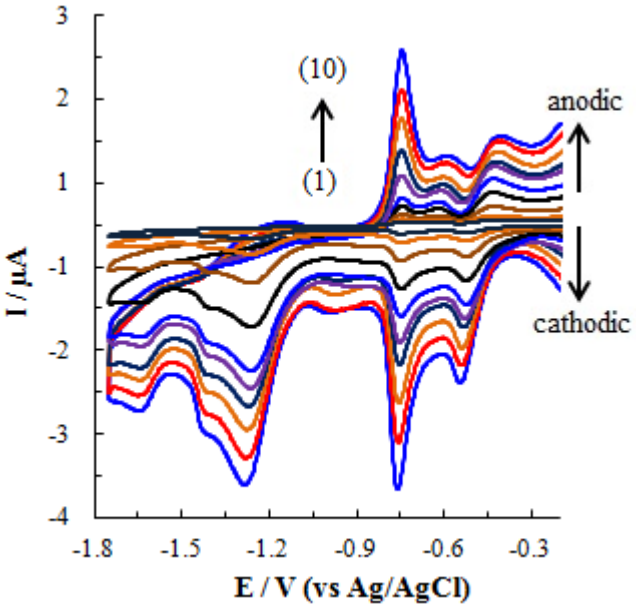


Figure 2. CVs of 0.5 mM ZFN solution with different scan rates in BR of pH 5.5 (scan rates in Vs^{-1}: (1) 0.03, (2) 0.05, (3) 0.10, (4) 0.18, (5) 0.25, (6) 0.35, (7) 0.50, (8) 0.65, (9) 0.80, (10) 1.00)

Electrochemical behavior was studied in detail. As a first step, effect of scan rate on peak potential was investigated while ZFN concentration was held constant as 0.5 mM. It is clear from the Fig. 2 that potential of reversible reduction-oxidation couple is independent of scan rate as expected for reversible nature and that of second reduction (PcII) and the third reduction (PcIII) were found to shift linearly to more negative values (shifted to more cathodic potentials) with logarithm of scan rate by the slope value of 45.2 mV and 24.2. Changing of peak potential with scan rate may be concluded as irreversible mechanism and charge transfer coefficient could be calculated from these slope values for second and third reduction mechanisms. According to literature [11, 13-16] slope of the curve plotted peak potential versus logarithm of scan rate is useful to calculate the multiplication of electron number and charge transfer coefficient. nα (n is number of electrons and α is charge transfer coefficient) for ZFN were calculated to be 0.95 for second reduction (PcII) and 0.50 for the third reduction (PcIII).

Effect of scan rate on peak current was also studied. Peak current of all peaks change linearly with potential scan rate (Fig. 3a) and current ratio of reversible reduction-oxidation couple (PcI-PaI) is

not affected by scan rate. Logarithm of peak currents were plotted versus logarithm of scan rate (Fig.3b) and linear relations were found as:

$$\text{Log(IpcI/A)} = 1.05 \log(v/Vs^{-1}) - 5.72;\ R^2 = 0.9989 \quad (1)$$

$$\text{Log(IpaI/A)} = 1.2 \log(v/Vs^{-1}) - 5.80;\ R^2 = 0.9985 \quad (2)$$

$$\text{Log(IpcII/A)} = 0.47 \log(v/Vs^{-1}) - 5.77;\ R^2 = 0.9947 \quad (3)$$

$$\text{Log(IpcIII/A)} = 0.49 \log(v/Vs^{-1}) - 6.63;\ R^2 = 0.9873 \quad (4)$$

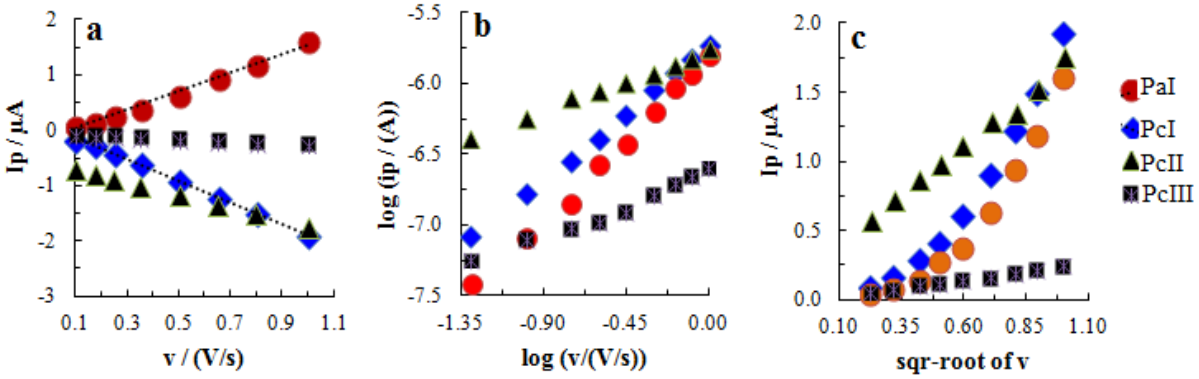


Figure 3. (a) Peak current vs. scan rate (b) logarithm of peak current vs. logarithm of scan rate (c) peak current vs. square-root of scan rate

According to literature, slope of logarithm of peak current versus logarithm of scan rate approaches to unity when mechanism is controlled by adsorption whereas diffusion controlled mechanisms have the slope value of 0.5 [13-20].

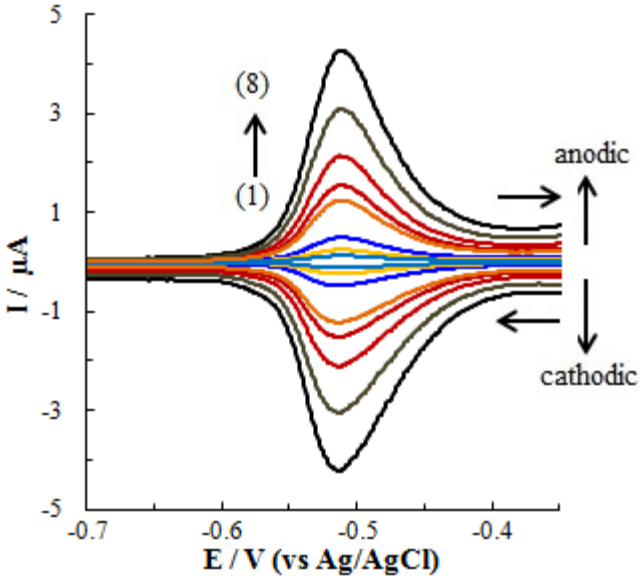


Figure 4. CVs of 0.05 mM ZFN solution between -0.2 and -0.7 V with different scan rates in BR of pH 5.5, (scan rates in Vs^{-1}: (1) 0.05, (2) 0.075, (3) 0.10, (4) 0.25, (5) 0.35, (6) 0.50, (7) 0.70, (8) 1.00)

Furthermore, peak current of reversible reduction-oxidation couple (PcI-PaI) is not changed linearly with square-root of scan rate where that for second reduction (PcII) and the third reduction (PcIII) has linear dependency (Fig. 3c). As a result, reversible couple should have surface confined properties and other reductions should have electrode-solution interface characteristics.

According to Fig.1 and Fig.2, ZFN seems to have two reduction and two oxidation peaks at potential region of reversible reduction-oxidation (PcI-PaI) couple when ZFN concentration is higher than 0.1 mM. Effect of ZFN concentration and effect of potential scan rate were detailed for this potential region and recognized that for ZFN concentration lower than 0.1 mM, there is only one reduction-oxidation couple in this potential region and it has nearly the ideal surface characteristics (i.e. potential of reduction and oxidation are nearly the mirror image of each other (Fig.4), peak currents and peak areas are nearly the same, peak current changes linearly with scan rate, logarithm of peak current changes linearly with that of scan rate by having the slope value of 1.0) as depicted in literature [13]. Because of its dependency of concentration and disappearance in low concentrations, they are concluded as pre or post peaks due to adsorptive characteristic.

3.1.2. Effect of pH

Afterwards, effect of pH on peak parameters were studied using square-wave voltammetry (SWV) between pH 2.0 and 11.0.

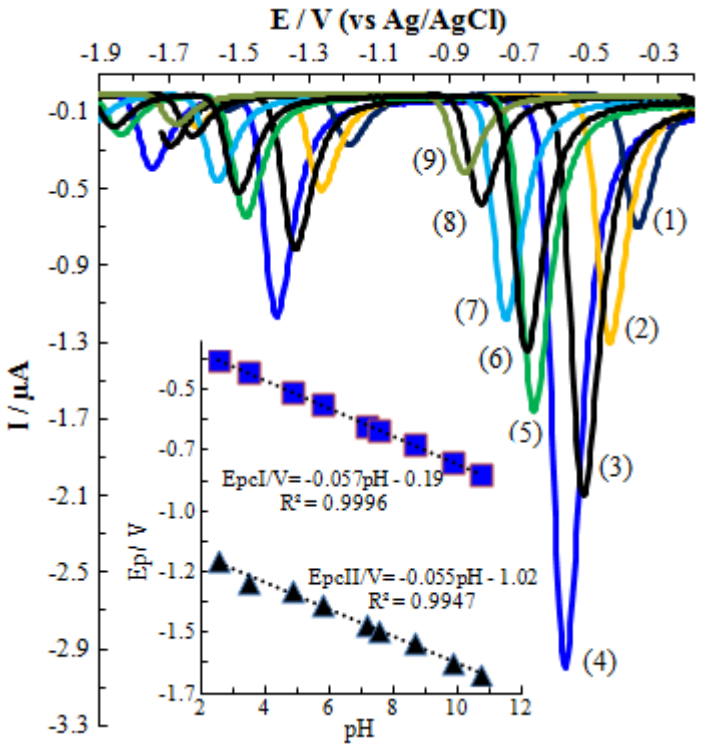


Figure 5. SWVs of 10.0 µM ZFN solution for different pHs (pH values: (1) 2.5, (2) 3.5, (3) 4.8, (4) 5.5, (5) 7.1, (6) 7.6, (7) 8.6, (8) 9.7, (9) 10.8, inset: peak potential vs. pH)

As could be seen from Fig. 5, potential of all reduction peaks (PcI, PcII and PcIII) shifted to more negative (more cathodic) potentials with increasing pH and that of PcIII shifts to out of suitable potential window when pH is in alkaline region. Changing of peak potential with the concentration of hydrogen ion is the evidence of participation of proton to mechanism. In pH studies, peak potentials for PcI and PcII were plotted against pH value (Fig.5 inset) and correlations between potential and pH were found as:

$$EpcI / V = -0.057pH-0.19; R^2 = 0.9996 \quad (5)$$

$$EpcII / V = -0.055pH-1.02; R^2 = 0.9947 \quad (6)$$

As could be understand from the slope values that these two reduction process are Nernstian and since slope of these graphs should be equal to $2.303RT\,\delta\,/nF$ where δ is the number of protons involved in the electrode reaction, n is the number of electrons transferred and the rest are commonly known constants [11, 19, 20] identical number of protons and electrons are participated in mechanisms. Peak current, peak shape and symmetry were taken into account and finally the optimum pH was selected as 5.5 for further studies.

3.1.3. Bulk Electrolysis (BE) and Number of Electrons

Constant potential BE studies were carried out at three different potentials. In the first (BE1) potential was hold on - 0.8 V, in the second (BE2) it was -1.55V and the last one (BE3) was carried out at -1.85 V. For each BE, 25 mL of 0.10 mM ZFN solution was used in BR of pH 5.5. Before and after BE, CVs of each solution were measured to control whether BE make any difference in both potential and current of peaks. In these studies, after BE, all peaks were disappeared and ratio of net charge for BE1:BE2:BE3 are found to be as: 2Q:4Q:5Q. As a results, ratio for number of electrons for PcI, PcII and PcIII should be as 2:2:1. By using Faraday's equations number of electrons were calculated as 2, 2 and 1 for the first reduction (PcI), for the second reduction (PcII) and for the third reduction (PcIII), respectively.

3.2. Theoretical Investigation

Electron(s) flow(s) from the electrode into the lowest unoccupied molecular orbital (LUMO) of the species when species was reduced. Consequently, location of LUMO is important to determine the most relevant part/atoms of the molecule for reduction reactions. It is therefore necessary to determine the HOMO-LUMO of the molecule to support the reduction mechanism in more accurate way. For this reason, in order to predict LUMO, ZFN geometry was optimized first, using semi empirical methods (AM1). Hence these methods are fast and often fail to predict accurate energy values of compounds, a more accurate basis set was found necessary to obtain energy values that match experimental accuracy. Accordingly, single point energy calculation processes were performed at B3LYP/6-31+G (d) and as a result HOMO and LUMO together with their corresponding energies are depicted in Figure 6.

According to Fig. 6, LUMO of ZFN is located around carbonyl groups and these groups recognized to be the most negatively charged part of ZFN. As reduction begins, at the first, protonation

of carbonyl group activated by vicinal nitrogen is considered and as a second reduction the other carbonyl group was suggested. For both reductions, firstly protonation of negatively charged part of molecule take place and this step is followed by electron transfer and finally carbonyl group was reduced to corresponding alcohol by totally 2H and $2e^-$. Prior to electron transfer, protonation of carbonyl oxygen is a classical acid catalyzed reaction. The reduction of carbonyl group will be more favorable at low pHs. Similarly, protonation step will be more difficult in higher pHs and higher potential will be needed as investigated in pH studies. Since third reduction is completely $1e^-$ and $1H^+$, catalytic reduction of proton that is activated by lone pair electrons in ZFN structure is proposed as shown in Scheme 2.

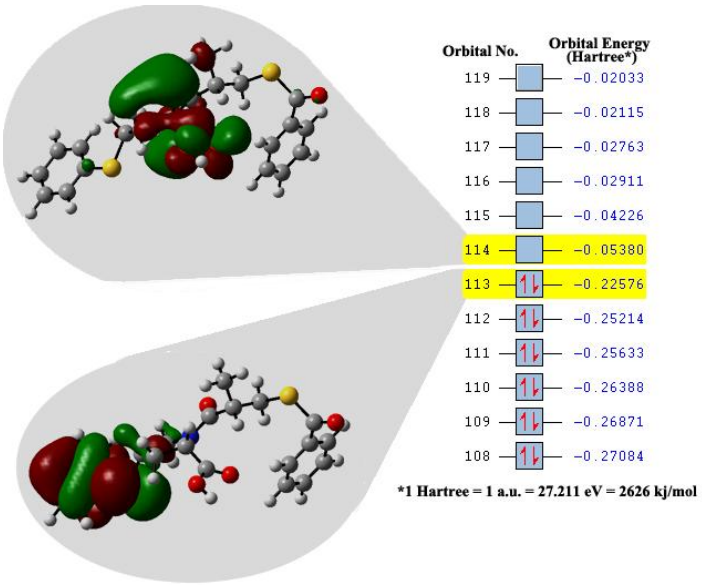
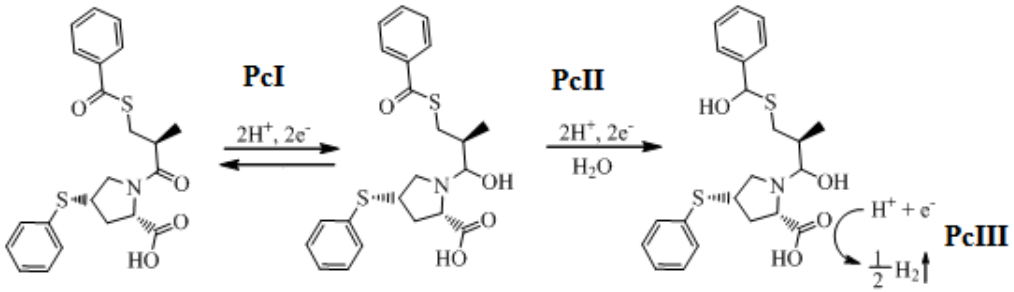


Figure 6. Frontier molecular orbitals mapped on optimized molecular structure of CTP, their corresponding energies calculated at B3LYP/6-31+G (d)//AM1



Scheme 2. Proposed reduction mechanisms for PcI, PcII and PcIII

3.3. Voltammetric determination of ZFN

In an effort to develop a voltammetric method for the determination of ZFN, quantitation of peak current were examined by square-wave (SWV) and differential pulse (DPV) techniques first

without using stripping mode. In such studies, SWV method was found to be more suitable and reproducible than DPV. Then, due to the adsorptive behavior, to get more sensitive method with lower detection limit, square-wave cathodic adsorptive stripping voltammetry (SWCAdSV) was applied.

3.3.1. Optimization of Variables

Nature of supporting electrolyte may affect the peak response in voltammetric studies. Thus, various electrolytes such as BR, phosphate and acetate buffer solutions were examined to find the optimum conditions. BR gave the highest peak current and better peak shape and it was selected for further studies. The effect of pH was also investigated. Peak current, peak shape and peak symmetry were taken into account and then optimum pH was selected as 5.5 as emphasized before.

Variation of peak current and its shape with instrumental conditions such as frequency (f), scan increment (ΔE_i), pulse height (ΔE), step increment (ΔE_i), accumulation time (t_{acc}), and accumulation potential (E_{acc}) was investigated using 0.5 µM ZFN at optimum experimental conditions. As a result, optimum instrumental parameters were found as follows: f = 25 Hz, ΔE_i = 3 mV, pulse width 0.01 s, ΔE = 50 mV, E_{acc} = 0.0 V and t_{acc} = 180 s.

Applying these optimized conditions, the applicability of the proposed voltammetric procedure for the determination of ZFN was investigated. Peak currents were measured as a function of ZFN concentration in quintuplicate under the optimized operational parameters and average of these five serial measurements was used as a peak current. Calibration graphs for ZFN were obtained to estimate the analytical characteristics of methods.

3.3.2. Validation of Proposed Methods and Determination of ZFN in Tablets and Human Serum

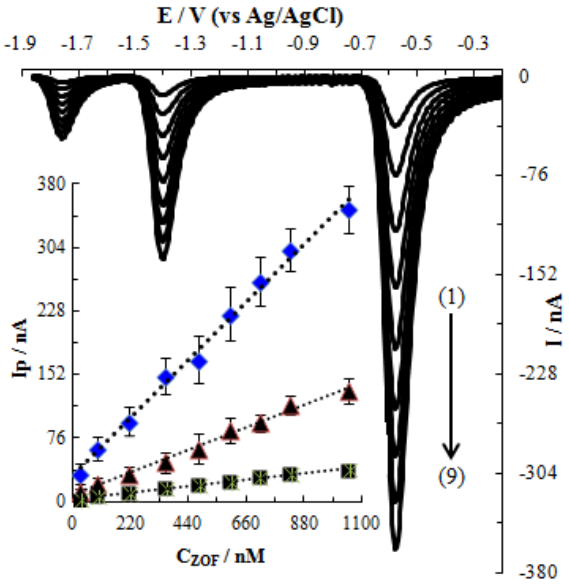


Figure 7. SWCAdSVs for calibration studies of ZFN at optimum conditions and calibration curves for PcI, PcII and PcIII, (concentrations: (1) 0.03, (2) 0.09, (3) 0.22, (4) 0.35, (5) 0.48, (6) 0.60, (7) 0.71, (8) 0.83, (9) 1.05 µM)

The proposed method was validated investigating the following parameters: Linearity range, sensitivity, limits of detection (LOD) and quantitation (LOQ), accuracy, reproducibility and repeatability according to ICH [21].

Linearity was checked by preparing standard solutions at more than ten different concentration levels. Five serial measurements were taken for each concentration and subsequent to evaluation of the required statistical test (Q-test), the average was used as a peak current of related concentration. Peak current of three reductions were found to change linearly with the ZFN concentration in the range from 0.03 µM (12.88 ppb) to 1.05 µM (451 ppb) and relation between peak current and ZFN concentration was found as given below for all reduction peaks:

$$IpcI / nA = (0.32 \pm 0.01)C_{ZFN} / nM + (28.08 \pm 1.15);\ R^2 = 0.9937 \quad (7)$$

$$IpcII / nA = (0.12 \pm 0.02)C_{ZFN} / nM + (7.74 \pm 0.92);\ R^2 = 0.9928 \quad (8)$$

$$IpcI / nA = (0.04 \pm 0.005)C_{ZFN} / nM + (3.02 \pm 0.25);\ R^2 = 0.9906 \quad (9)$$

The good linearity of the calibration graphs and the negligible scatter of the experimental points are clearly evident from the coefficient of determination (R^2) (eqs 7-9 and Fig.7).

Slope value of corresponding calibration (dIp/dC) was considered as the sensitivity of proposed method. Accordingly, PcI has the highest sensitivity towards ZFN, sensitivity of PcII is higher than PcIII.

LOD and LOQ values were calculated using equations given in the literature [11, 19, 20], and results (for PcI) with some other validation parameters are given in Table 1.

Table 1. Regression data of the calibration curve

Regression Parameter	PcI
Linearity range / µM	0.03-1.05
Slope of calibration curve / ALmol^{-1}	0.32
Intercept / µA	0.03
Standard deviation (SD) of regression / nA	9.6
SD of slope / µALmol^{-1}	0.01
SD of intercept / nA	1.15
Limit of detection (LOD) / µM	0.01
Limit of quantification (LOQ) / µM	0.033
Determination coefficient, R^2	0.9937
Within-day repeatability of peak current*, (% RSD)	6.79
Between-day repeatability of peak current*, (% RSD)	8.96
Within-day repeatability of peak potential*, (% RSD)	2.78
Between-day repeatability of peak potential*, (% RSD)	3.98

**for 5 serial measurement*

In order to evaluate the applicability of the proposed method to pharmaceutical preparations and biological samples, ZFN was determined in Zoprotec® tablets and spiked human serum samples. As shown in Table 2, mean results of each application lie around 96.9 % (RSD < 10.0 %) for tablet recovery. This result indicates the validity of proposed method for ZFN assay from tablets.

Table 2. Recovery of ZFN from Zoprotec® tablets

Nominal value, mg	Values calculated, mg	Recovery*, %	RSD**, %
30.0	25.8; 27.1; 28.6; 30.9; 32.9	96.9 ± 11.8	9.8

**value = average ± ts/√N (N=5 and at 95 % confidence level)*
***relative standard deviation for 5 serial measurements*

Recovery studies in spiked human serum samples were also performed. In these applications, voltammetric base line for ZFN-free serum samples in BR solution was taken and no voltammetric signal in the potential range of ZFN was found. It was concluded that there is no interference effect of any potential species found in human serum. As could be seen in Table 3, recovery values are around 97.7 % and 101.4 %. The differences between spiked and calculated concentrations are insignificant at 95 % confidence level.

Table 3. Recovery of ZFN from spiked human serum

Value Spiked, µg	Value calculated, µg	Recovery*, %	RSD**, %
1.0	0.86; 0.97; 1.00; 1.00; 1.06	97.7 ± 9.2	7.6
3.0	2.71; 2.76; 3.15: 3.29; 3.30	101.4 ± 11.9	9.4

**value=average±ts/√N (N=5 and at 95 % confidence level)*
***relative standard deviation for 5 serial measurements*

4. CONCLUSION

Electrochemical reduction characteristics of ZFN on HMDE with the help of computational calculations were studied for the first time. Redox properties and electrochemical parameters of drug molecules may be important for understanding the mechanism of action and their target/related organs. Combining CV, BE and theoretical calculations together, the electrochemical reduction of CTP was proposed to occur at carbonyl groups. Determination of drug molecules in pharmaceuticals and biological samples are also of great importance. In the present study, precise, accurate, rapid and sensitive methods which require neither sophisticated instrumentation nor tedious extraction processes have been proposed. Consequently, the proposed methods have the potential of a good analytical alternative for determining ZFN in pharmaceutical formulations and human serum. Also, they can be adopted for pharmacokinetic studies as well as for quality control laboratory studies.

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