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NON-EXTRACTIVE ULTRA-TRACE DETERMINATION OF SIMVASTATIN IN BIOLOGICAL FLUIDS BY VOLTAMMETRIC METHOD VIA COMPLEXATION WITH CADMIUM

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The aim of the present study was to develop a rapid, non-extractive and sensitive square-wave adsorptive stripping voltammetry method for the quantitative analysis of simvastatin (SIM) in biological fluids by complex formation with Cd (II). The experimental parameters affecting the response of the SIM-Cd (II) complex were carefully investigated and optimized. This method was based on electrochemical reduction of SIM-Cd(II) complex at a hanging mercury drop electrode in Britton-Robinson buffer (pH 2.5) containing Cd(II). The cyclic voltammetry studies indicated that the reduction process was irreversible and primarily controlled by adsorption. A well-defined reduction peak was observed at -1000 mV with 30 s accumulation time and 0.0 V accumulation potential versus silver reference electrode. The developed method met ICH regulatory requirements. The AdSV peak current was proportional over the concentration range $1x10^{-6}$ -$1.5x10^{-5}$ M, ($r = 0.999$), with limit of detection of $2.2x10^{-10}$ M ($n = 8$). The proposed electrochemical procedure was successfully applied to the determination of SIM in human plasma and urine with mean recoveries of 85 ± 1.41% and 87 ± 1.23%, respectively. No electroactive interferences from endogenous substances were found in biological fluids.

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Keywords: Adsorptive stripping voltammetry; Cyclic voltammetry; Simvastatin-Cd (II) complex; Biological fluids

1. Introduction

Adsorptive stripping voltammetric technique, especially with the square-wave waveform, is an extremely simple and sensitive technique that can be used for analysis of drugs without the requirement for extraction steps prior to the assay. Moreover, square-wave voltammetry is a large amplitude differential technique in which a waveform composed of a symmetrical square wave is applied to the working electrode. The current is sampled twice during each square-wave cycle, once at the end of the forward pulse and once at the end of the reverse pulse. The resulting peak current is proportional to the concentration of the analyte. Excellent sensitivity accrues from the fact that the net current is larger than either the forward or the reverse components current. Coupled with the effective discrimination against the charging current, very low detection limits can be attained. However, many of the adsorptive stripping voltammetric (AdSV) approach features such as sensitivity, selectivity, simplicity and versatility attributed to the combination of an effective preconcentartion step based on non-electrolytic adsorptive accumulation process with

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advance measurement procedures such as deferential pulse (DP) or square wave (SW) [1-5]. In addition, square wave - adsorptive stripping voltammetry (SW-AdSV) has been characterized as an extremely sensitive source for electrochemical measurements since its establishment half a century ago. Such electrochemical approach with improved sensitivity and selectivity has promoted the development of numerous analytical applications of ultra-trace determinations of a variety of organic and inorganic substances, like a SW-AdSV method which involves a stripping step that was carried out by using a square wave time-potential waveform imposed on the working electrode. The most important advantages of SW-AdSV over other AdSV techniques (namely differential pulse and linear sweep) are its enhanced detection capabilities, speed of analysis and the possibility to avoid the oxygen removal step in the analysed samples [1,6,7]. Several reviews were devoted to emphasize and illustrate the wide spectrum and scope of SW-AdSV applications and potentialities in the analysis of metal ions [8, 9], organic analytes [10] and pharmaceutical compounds [11-13]. Many analytical reviews also were used to determine organic compounds and nanomaterials [14-26]. Moreover, several voltammetric methods such as square wave voltammetry and differential pulse voltammetry were used to analysis of drugs and different organic compounds [27-34].

Simvastatin, (1*S*,3*R*,7*S*,8*S*,8*aR*)-8-{2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate, belongs to a class of drugs called statins, which act by inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. This microsomal enzyme catalyzes the rate-limiting step of cholesterol biosynthesis. Also, SIM inhibits oxidation of native and modified low-density and high-density lipoproteins [35]. Statins are currently the most therapeutically effective drugs available for reducing LDL cholesterol and triglyceride levels in the blood stream of patients at risk of cardiovascular disease [36] even they are now among the most frequently prescribed drugs [37]. The chemical structure of SIM contains a β-hydroxy- lactone so the physiologically active form of the drug is the β-hydroxy acid, which is formed by a ring-opening reaction of the lactone ring. *In vivo*, prodrug lactone form is enzymatically hydrolysed to its hydroxyl-acid pharmacophore [38].

Developments of sensitive and selective analytical methods to the determination of drugs are highly required. SIM has been determined in pharmaceutical preparations and biological fluids using several analytical methods included; high-performance liquid chromatography (HPLC) combined with mass spectrometry [39-42] or fluorescence [43] or UV detection [44]. Moreover there are several methods for analysis of SIM involved gas chromatography (GC) [45], capillary electrophoresis (CE) [46] and UV spectroscopy [47]. However, some of these methods required expensive equipment [39-42] and are time-consuming [46, 47].

Two methods only are reported in the literature featuring the voltammetric determinations of SIM at a glassy carbon electrode in 0.1 M H_2SO_4 [48] and at different buffers by use cathodic square wave voltammetry (CSWV) [49]. These methods had a drawback of low sensitivity and used different buffer solutions. For these reasons, the development of new alternative analytical method for determination of SIM in biological fluids with adequate sensitivity, improved simplicity was seriously needed. The present study describes for the first time, the development and validation of a sensitive voltammetric method for the direct determination of SIM-Cd (II) complex in human urine and plasma based on the reduction of SIM-Cd(II) complex at a hanging mercury-drop electrode.

2. Experimental

2.1 Apparatus

All voltammetric measurements were carried out with 797 VA computrace (Metrohm, Switzerland) in connection with Dell computer (Chinese made) and controlled by VA computrace 2.0 control software. A conventional three electrode system was used in the hanging mercury drop electrode (HMDE) mode inside of Ag/AgCl, KCl (sat) reference electrode and Pt auxiliary electrode. Nitrogen cylinder grade five (Hashim company-SA) was used and connected in the

voltammetric system. pH values were measured with Hanna instruments pH211 (Romania made) pH meter.

2.2 Reagents

Simvastatin was obtained from Sigma Chemical Co. (St Louis, MO, USA). Other chemicals used were of analytical reagent grade and were used without further purification. SIM stock solutions of 1×10^{-2} M were prepared by dissolving the appropriate amount of this compound in distilled water in 25 ml volumetric flask. These stock solutions were stored in the dark and under refrigeration in order to minimize decomposition. Standard solutions of this compound with lower concentrations were prepared daily by diluting the stock solution with distilled water. Cadmium (II) stock solution of 1.0×10^{-3} M was prepared by dissolving the appropriate amount of $Cd(NO_3)_2.4H_2O$ in distilled water in 25 ml volumetric flask. Britton-Robinson supporting buffer (pH ≈ 2, 0.04M in each constituent) was prepared by dissolving 2.47g of boric acid (made in UK, winlab) in 500 ml distilled water containing 2.3 ml of glacial acetic acid (UK, BDH) and then adding 2.7 ml of ortho-Phosphoric acid (Germany, Riedal-deHaen) and diluting to 1 L with distilled water. In addition, phosphate supporting buffer [0.1 M NaH_2PO_4 and 0.1 M H_3PO_4] was prepared by dissolving 12 g of NaH_2PO_4 (UK, BDH) and 6.78 g of H_3PO_4 in 1000 ml distilled water. Acetate supporting buffer (0.02 M in each constituent) was prepared by dissolving 1.68 g of sodium acetate (UK, BDH) in 500 ml distilled water containing 1.12 ml of acetic acid and diluting to 1 L with distilled water. Finally, carbonate supporting buffer (0.1 M in each constituent) was prepared by dissolving 10.6 g of sodium carbonate (UK, BDH) and 8.4 g of sodium hydrogen carbonate (UK, BDH) in 1L distilled water.

2.3 Procedure

2.3.1 Optimization step

Supporting electrolyte (10ml) was placed in the electrochemical cell and the required aliquots of standard SIM and Cd(II) solutions were added by a micropipette. The prepared solution was deoxygenated prior to analysis by purging with purified nitrogen for 5 min with the stirrer on. The samples were blanketed with nitrogen during acquisition. After forming a new mercury drop, the square-wave voltammogram was recorded either immediately in quiescent solution or after adsorptive accumulation for a selected time at the predeter-mined potential in stirred solution. A quiescent period of 10 s was allowed before commencing the potential scan. The voltammetric scans were carried out over the range 0.0 to -1.3V. All measurements were carried out at the room temperature.

2.3.2 Analysis of SIM in Spiked Human Plasma and Urine

Accurately measured aliquots of SIM solutions were pipette into centrifugation tubes containing 500 µL human plasma and/or urine, and then the sample centrifuging was continued for 5 min. Into each tube, 1.0 ml of ethanol, 0.1 ml NaOH (0.1M), 1.0 ml $ZnSO_4.7\ H_2O$ (5% w/v) were added then centrifuged for 30 min at 3500 rpm [50]. The clear supernatant layer was filtered through 0.45µm Milli-pore filter. A 0.1 ml volume of the supernatant liquor was transferred into the voltammetric cell then completed to a 10 ml volume with a pH 2.5 B–R universal buffer. Then SIM was quantified by means of the proposed stripping voltammetric procedure.

3. Results and discussion

3.1 The Electrochemical Behavior of SIM

Preliminary stripping voltammetric experiments shown that SIM molecule undergoes reduction at the mercury electrode as can be expected from previous voltammetric studies [49]. As well-known, the voltammetric signal of cadmium metal is fixed and recorded at E_p= -0.55V and the voltammetric current of SIM was also recorded at E_p = -1.4V. While in this work, the stripping voltammetric peak of SIM-Cd(II) complex was observed at E_p= -1.00V by use pH 2.5 B-R buffer also the SW-AdSV behavior of the studied complex was shown in Figure 1. However, the addition of SIM and Cd (II) concentrations to the previous test solution provided a well-defined cathodic peak (versus Ag/AgCl reference electrode). In fact, the cadmium ion exhibited a good affinity towards SIM molecule forming a very stable SIM-Cd(II) complex which is strongly adsorbed onto the HMDE surface. This obtained stripping voltammetric peak was found to response sharply to the addition of either SIM or Cd (II) concentrations (lines B, C and D), which probably reflect the formation and adsorption of the suggested complex.

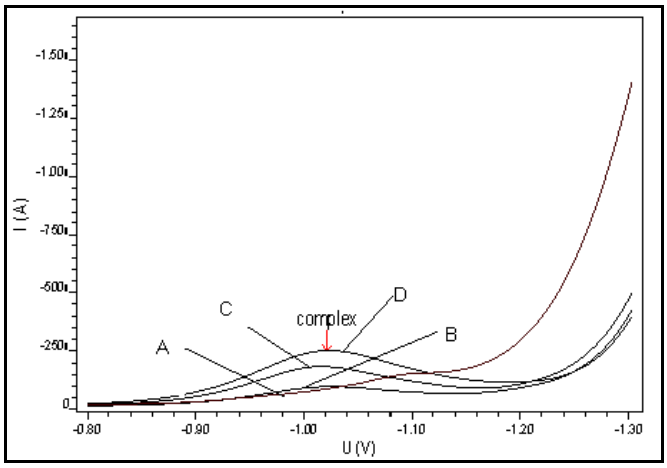
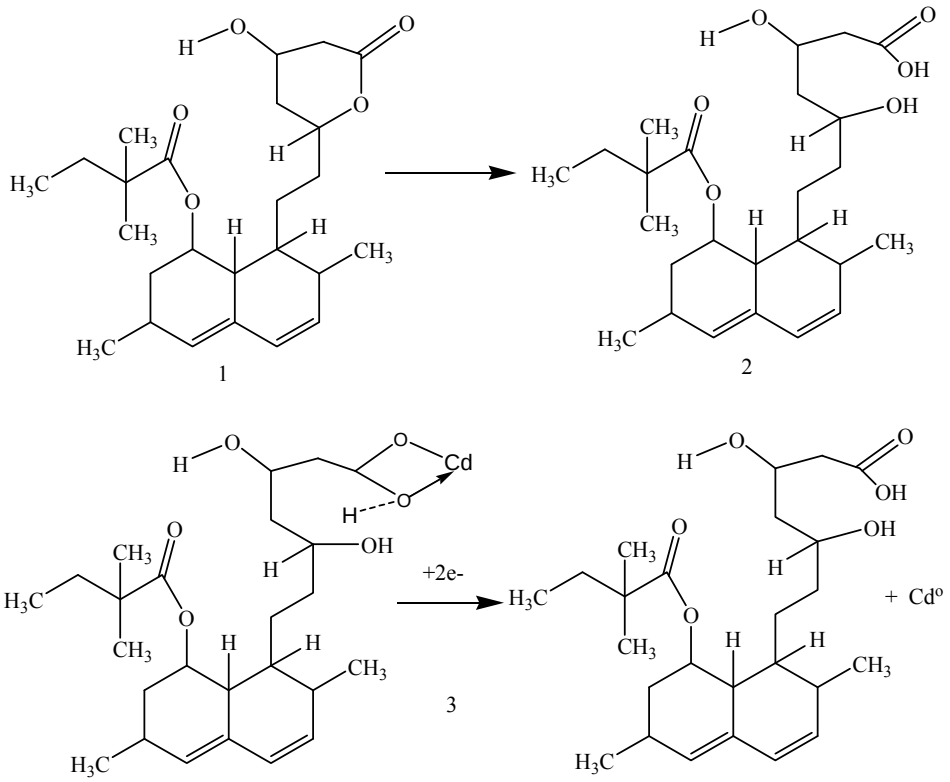


Fig. 1. Electrochemical behavior of simvastatin-Cd(II) complex: A = buffer + 1x10^{-5} M Cd(II), I(-nA)=0.0, B = buffer + Cd(II) + 5x10^{-6} M simvastatin, I(-nA)= 50.4, C = A + B + 2x10^{-5}M Cd(II), I(-nA)= 123, D = A +B +C + 3x10^{-5}M Cd^{+2}, I(-nA)=177, at pH 2.5 B-R buffer.

The observed AdSV peak is most probably due to the cathodic reduction of Cd (II) in the adsorbed complex with SIM and the electrochemical mechanism of this reduction process for SIM-Cd (II) complex was illustrated in scheme 1, inside of the molecular structure of simvastatin and the physiologically active simvastatin b-hydroxy acid form [49].



*Scheme 1. Molecular structure of simvastatin (**1**), physiologically active simvastatin β-hydroxy acid form (**2**) [49] and the proposed mechanism for the reduction process of simvastatin-Cd(II) complex (**3**)*

Reduction-oxidation chemical behavior of SIM-Cd(II) complex was investigated by using cyclic voltammetry (CV) technique. The voltammogram peak of $1x10^{-4}$M SIM ($1x10^{-3}$M Cd(II)) complex was obtained (I (-nA)= 1530) at B-R buffer and pH = 2.5 as shown in Figure 2. It was attributed to the electrochemical reduction of Cd^{++} ion in the complex to Cd^{o}. The observed cyclic voltammogram confirmed the irreversibility nature of the electrochemical process.

On the other hand, from the using of multi-cyclic voltammetry technique for $1x10^{-4}$ M SIM-Cd(II) complex ($1x10^{-3}$ M Cd(II)) at B-R buffer, pH= 2.5, 50 mV/s scan rate and five sweep rates, it was noticeable that, a high current was observed in the first sweep. Then, the analyzed complex was accumulated on the surface of working electrode (HMDE) which let to drastic decrease and constant of the current.

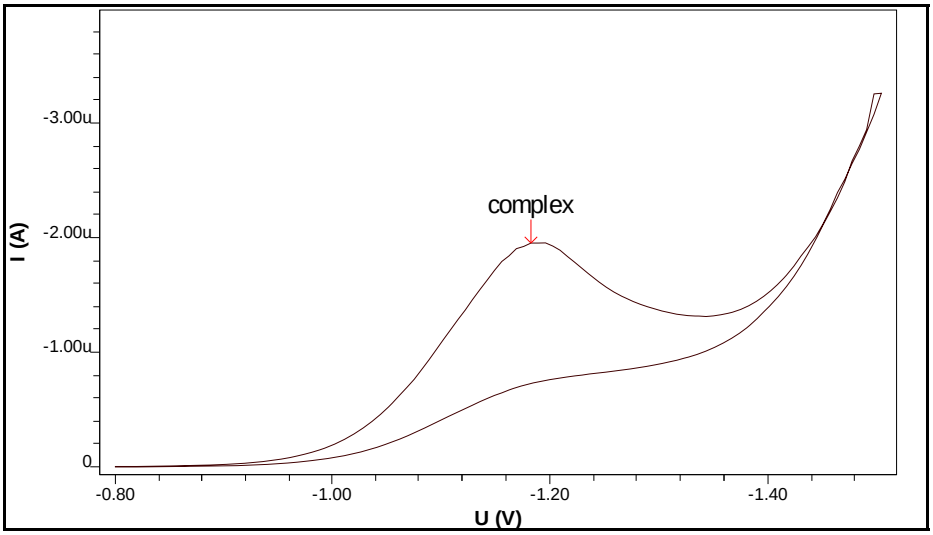


Fig. 2. Cyclic voltammogram of simvastatin-Cd(II) complex 1x10^{-4} M (1x10^{-3}M Cd(II)) at B-R buffer pH=2.5, 50mV/s scan rate.

In the case of using high sensitive AdSV-SW as a developed technique, a voltammetric peak is recorded at HMDE for 5x10^{-6} M SIM and 5x10^{-5} M Cd(II)-complex in B-R buffer, pH 2.5 and in the best conditions as given in Figure 3, which illustrates a well observed electrochemical peak indicating a strong and readily adsorption process at the surface of the working electrode. This can be attributed to the reduction through the electroactive Cd^{+2} moiety which supported the proposed mechanism. These results motivated us to go deeply for further investigation of different parameters.

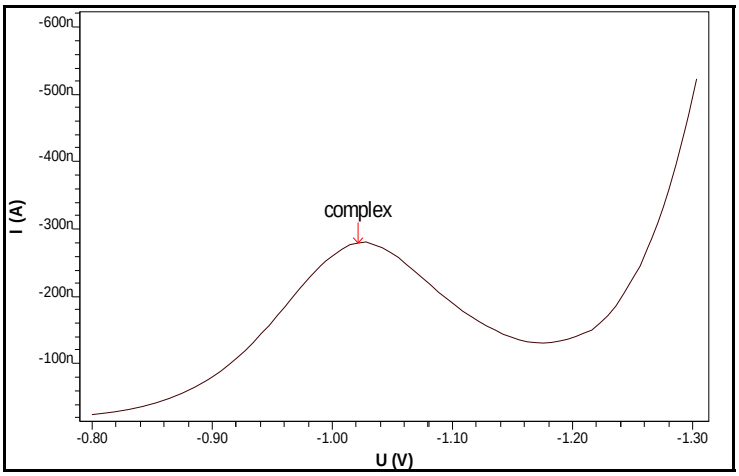


Fig. 3. SW-AdSV voltammogram (I(-nA)=193.33) of the (1x10^{-6} M simvastatin - 5x10^{-5} M Cd^{+2}) complex in B-R buffer, pH= 2.5, t_{acc} =30sec and E_{acc}=0.0V.

3.2 Parameters affecting SW-AdSV response

3.2.1 Influence of supporting electrolyte and pH

In order to study the effect of supporting electrolyte on the reduction process of the complex, B-R buffer of pH 3, pH 7, pH10, acetate buffer of pH 3, phosphate buffer pH 3 and carbonate buffer pH 10 with concentration of 5x10^{-6} SIM-2x10^{-5}Cd(II) M complex were examined. In term of sensitivity and the peak sharpness, the best cathodic reduction signal was

detected when B-R buffer pH 3 used. The optimal pH value was refined by changing the pH of the B-R buffer in the range 2.0-6.0: as a result, a pH of 2.5 was selected.

3.2.2 Influence of accumulation time and potential

One of the most essential conditions for highly sensitive determinations is the accumulation of the complex on the surface of the working electrode (HMDE). This study showed that the optimal signal was obtained for a deposition potential of 30 s when a concentration $5x10^{-6}$ SIM in the presence of $2x10^{-5}$ Cd^{2+} M was used (Fig. 4).

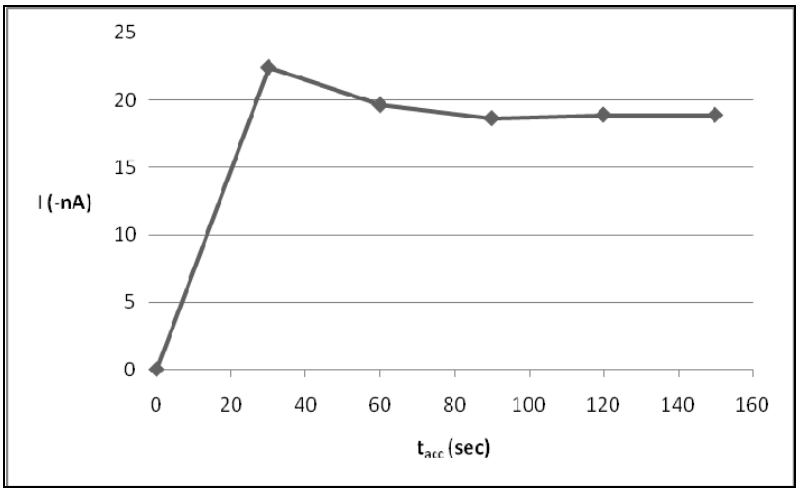


Fig. 4. Influence of accumulation time (t_{acc}) on the AdSV peak current of $5x10^{-6}$ M simvastatin- $2x10^{-5}$ M Cd^{+2} complex at pH2.5 B-R buffer.

For the forthcoming experiments, an accumulation time of 30s was selected as the best conditions due to the representing of the highest current value. Furthermore, variation of the accumulation potential over the range from -0.8 V to +0.8 V at 30 sec accumulation time as shown in Figure 5, revealed that a preconcentration potential of 0.0 V was the ideal choice for optimal sensitivity.

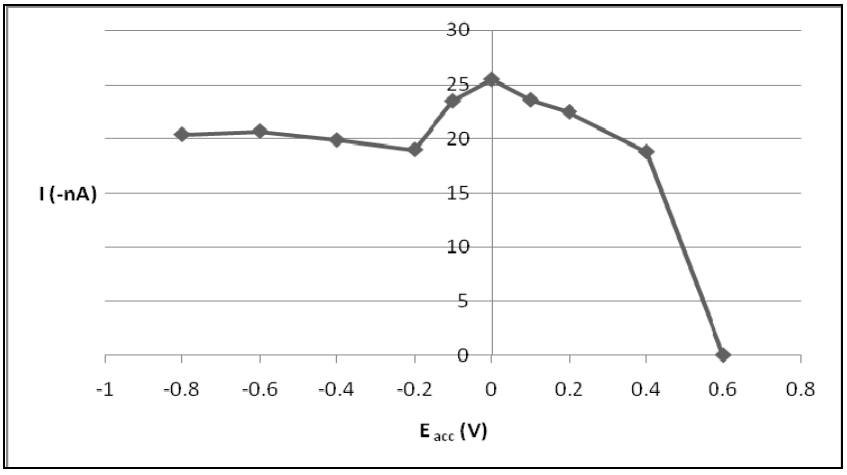


Fig. 5. Influence of accumulation potential (E_{acc}) of $5x10^{-6}$ M simvastatin- $2x10^{-5}$ M Cd^{+2} complex at pH 2.5 B-R buffer and t_{acc} =30 sec.

3.2.3 Influence of Cadmium concentrations

The dependence of the SW-AdSV voltammetric current of 5 $\times 10^{-6}$ M SIM in a B-R buffer of pH 2.5 on the concentration of Cd^{2+} ions was also investigated. As shown in Figure 6 the monitored voltammetric signal was approximately linear over the range from 1 $\times 10^{-6}$ M to 5 $\times 10^{-5}$ M Cd (II). Anyway, 2x10^{-5} M or 5 x10^{-5} M was given a good result for finding the SIM-Cd(II) complex.

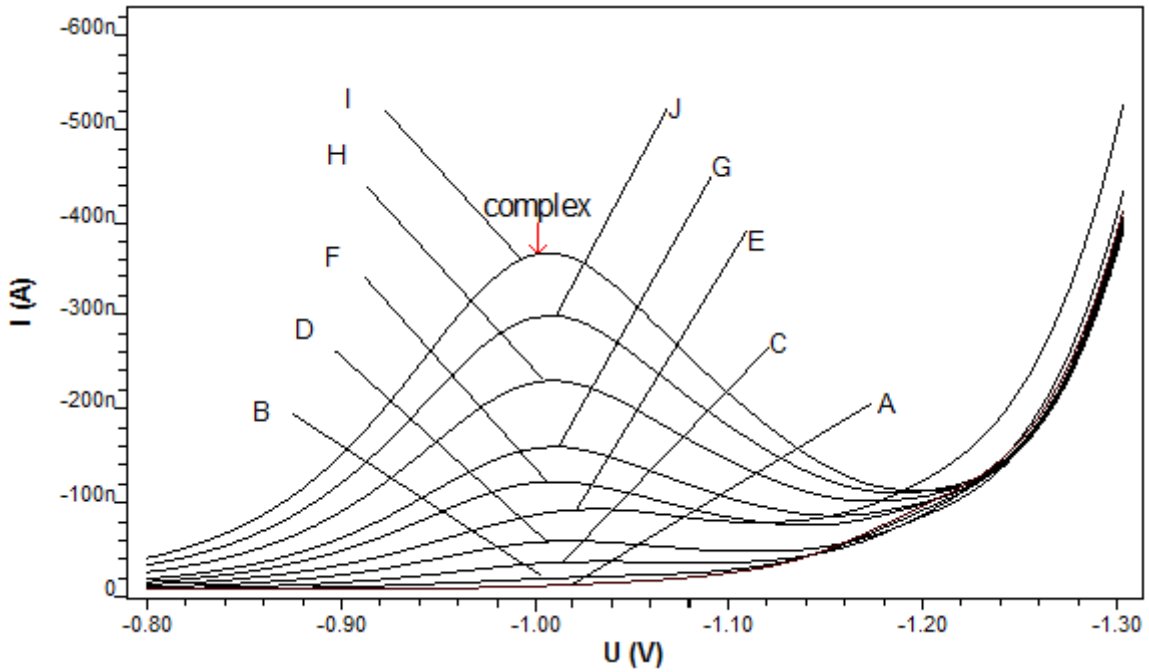


Fig. 6. Influence of Cd^{+2} concentrations of the complex voltammetric current (5x10^{-6} M simvastatin) at pH2.5 B-R buffer, [A=5x10^{-6} M simvastatin, B=A+1x10^{-6}M Cd^{+2}, C,D,E,F,G,H,J and I = 3,6,9 x10^{-6} and 1.5,2,3,4,5 x10^{-5} M Cd^{+2}, respectively].

3.2.4 Influence of potential sweep conditions

Generally, the SW-AdSV response depends on various parameters related to the way the applied potential was scanned. For instance, the cathodic peak current of the complex was found to be proportional to the scan rate, particularly at low scan rate values, as expected for adsorbed species [51]. As shown in Figure 7, the alteration of scan rate between 10 and 300 mVs^{-1}, caused the SW-AdSV peak current to increase gradually over the range 10-300 mV/s, but the shape of the voltammetric peak was not be good after 200 mV/s. Hence, 200 mV s^{-1} scan rate was selected for the subsequent works, because it ensured adequate sensitivity with short practical time.

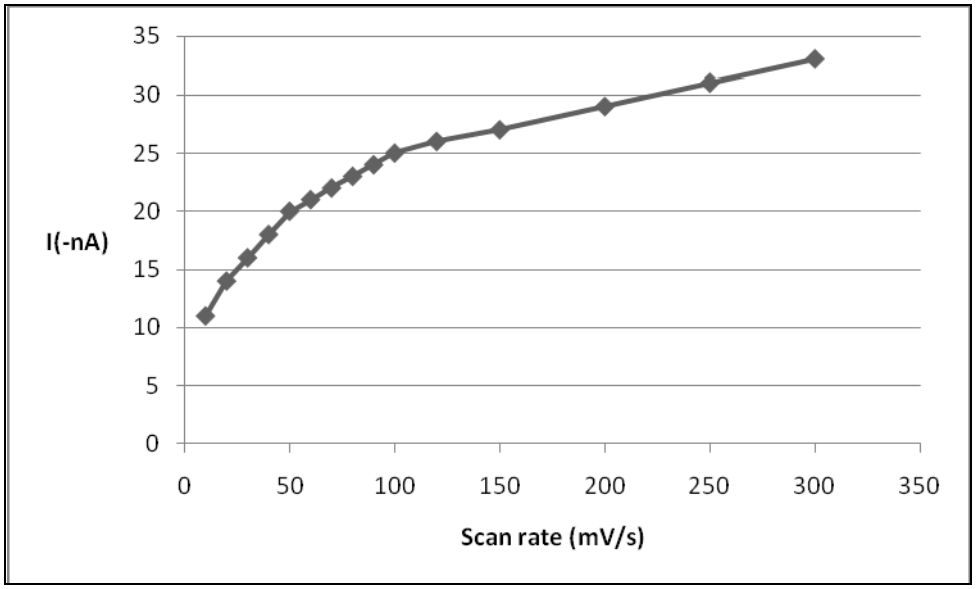


Fig. 7. Influence of scan rate of $5x10^{-6}$ M simvastatin- $2x10^{-5}$ M Cd^{+2} complex at pH2.5 B-R buffer, t_{acc} =30 sec and E_{acc}=0.0V.

Furthermore, the influence of changing the pulse amplitude on the square-wave voltammetric current was also evaluated over the range 10-100 mV as shown in Figure 8. The peak current of the complex almost increased linearly with pulse amplitude over the range 10-80 mV and at higher values than 80 mV the peak current reached a plateau.

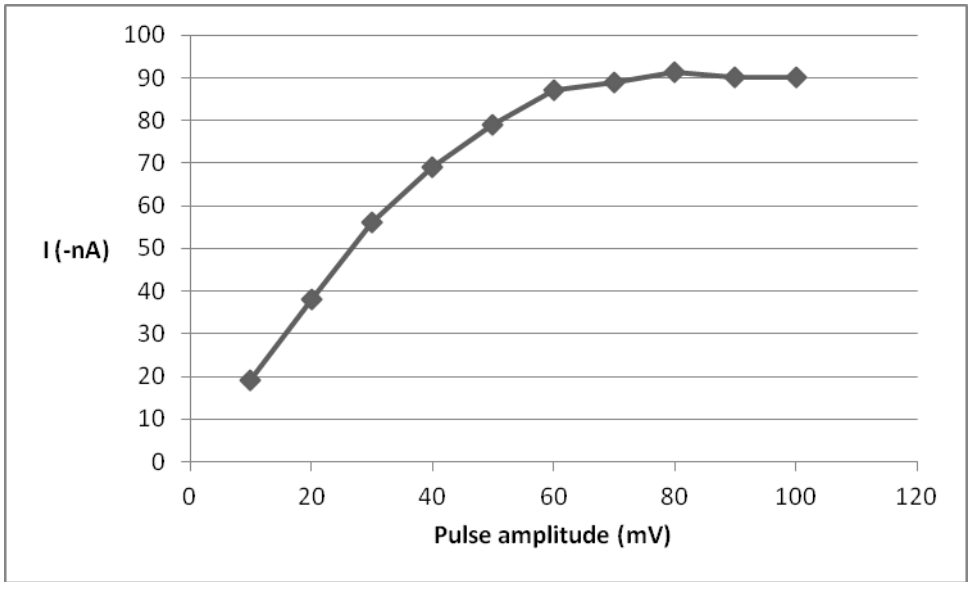


Fig. 8. Influence of pulse amplitude of $5x10^{-6}$ M simvastatin- $2x10^{-5}$ M Cd^{+2} complex at pH=2.5, B-R buffer, t_{acc} =30 sec, E_{acc}=0.0V and 200 scan rate.

Accordingly, 80 mV pulse amplitude was adopted as optimum value. In order to estimate the influence of square wave frequency on stripping voltammetric peak current, the value of this parameter varied over the range 5-40 Hz as given in Figure 9. The voltammetric signal of the complex was increased with the studied frequency range, but the best signal was found at 30 Hz,

after this value the shape of signal was bad. Hence, for further studies a 30 Hz frequency was chosen.

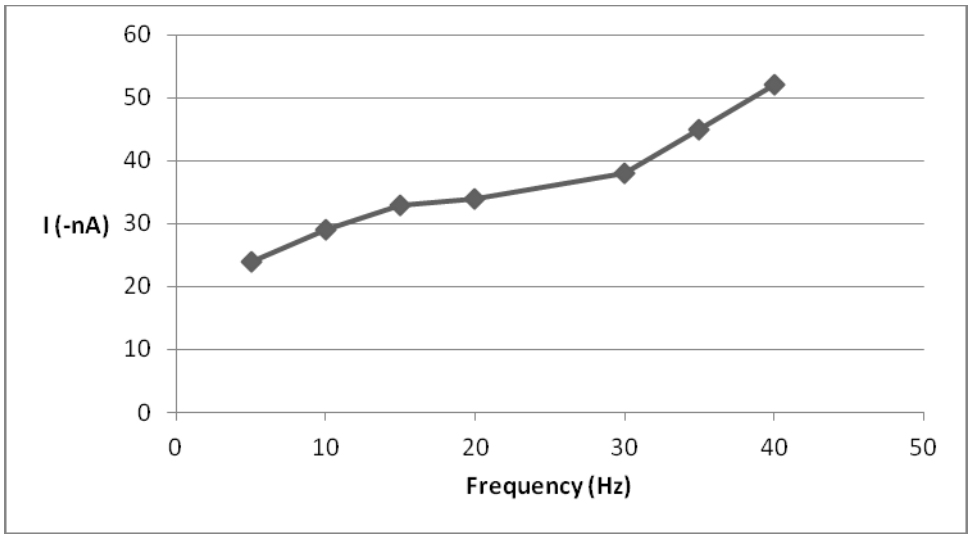


Fig. 9. Influence of the frequency of 5x10^{-6} M simvastatin- 2x10^{-5} M Cd^{+2} complex at pH = 2.5, B-R buffer, t_{acc} =30 sec, E_{acc}=0.0V, 200 scan rate and 80mV amplitude.

3.2.5 Influence of instrumental parameters

The observed electrochemical current can be further enhanced by optimizing other instrumental factors that can influence the adsorption accumulation process of the SIM-Cd(II) complex. Increasing the mercury drop surface over the range 0.15-0.60 mm^2 yielded, as expected, an enhancement in the stripping voltammetric peak current. As a matter of fact, the peak current of the complex was increased linearly with working electrode area over the range 0.15-0.60 mm^2 as shown in Figure 10. Hence, 0.60 mm^2 mercury drop size was considered as optimum valve. Similarly, the adsorptive stripping peak current can be maximized by selecting faster stirring rate, yet, to reach a largest possible amount of the studied material to a working electrode surface, a moderate 3000 rpm stirring speed was chosen as optimum value.

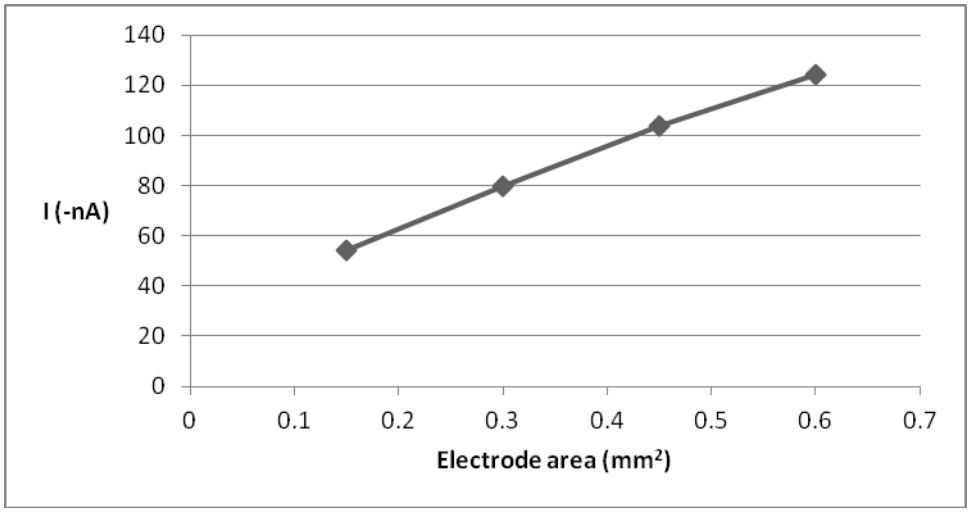


*Fig. 10. Influence of electrode surface area of 5x10^{-6} M simvastatin- 2x10^{-5} M Cd^{+2} complex at pH=2.5, B-R buffer, t_{acc} =30 sec, E_{acc}=0.0V, 200 scan rate, 80mV amplitude and 30*Hz.

3.3 Validation of the Method

3.3.1Calibration graph

Under the optimum experimental conditions a good linear correlation was obtained between SIM-Cd(II) complex electrochemical response and its concentration in the range 1×10^{-6}- 1.5×10^{-5} M SIM ($5x10^{-5}$ M Cd^{+2}), [Figure 11]. The parameters of the complex concentration-current straight line were calculated by the least-squares method. The regression equation of the calibration line has the form:

$$i_p\,(nA) = 1.66\times10^{7}\,C\ (M) + 103.3 \qquad r = 0.999 \quad n = 6\ .$$

where i_p is the SW-AdSV peak current, C is the SIM-Cd^{++} concentration and r is the correlation coefficient.

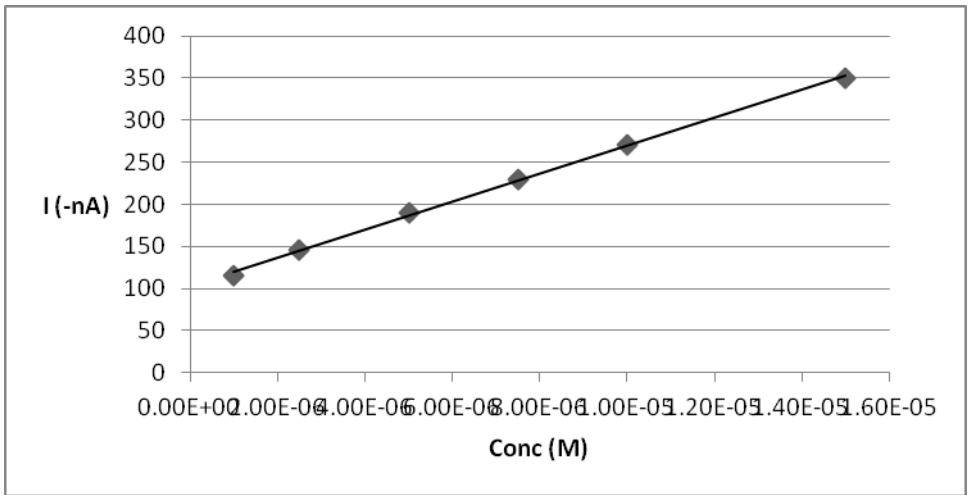


Fig. 11. The calibration curve of the simvastatin-Cd(II) complex.

3.3.2 Detection Limit

The detection limit, defined as three times the signal-to-noise ratio (S/N =3) reached in the optimum conditions for monitoring this SIM-Cd(II) complex was 2.2×10^{-10} M. Such remarkable sensitivity illustrates the preference of this electrochemical technique over the other voltammetric techniques of SIM determination, which achieved 2.71,5.5 x 10^{-7} M and 4.5 x 10^{-9} $moll^{-1}$ detection limit [48,49]. Obviously, the applied stripping voltammetric approach enhanced the sensitivity by 2-3 orders of magnitude in contrast to the cited analytical methods.

3.3.3 Reproducibility

The analytical precision of the developed method was verified from the reproducibility of 8 determinations of 5×10^{-6} M SIM and $2x10^{-5}$ M Cd^{+2} complex in B-R buffer at pH 2.5. A relative standard deviation (RSD) of 3.66 $x10^{-3}$ % was calculated, which indicates reproducible accumulation and monitoring of the studied complex.

3.3.4Recovery

The recovery of the developed procedure, which reflects the accuracy of the method, was evaluated by analyzing spiked buffer solution containing 2×10^{-6} M SIM- $5x10^{-5}$ M Cd^{+2} complex via the optimized SW-AdSV procedure. The mean recovery of four measurements was found to be 97% ± 2.16.

3.3.5 Stability

The stability of 5×10^{-6} M SIM- $5x10^{-5}$ M Cd^{+2} complex solution was investigated by monitoring the SW-AdSV signal at the optimum analytical conditions every ten minutes and the measured electrochemical response seemed to be nearly fixed over the studied time period 0-80 minutes.

3.4 SW-AdSV Analytical applications

The developed SW-AdSV method has been applied to the determination of the complex in some real samples such as human urine and plasma, by estimating its recovery from these biological samples. A simple and fast pretreatment (clean-up) procedure, which is in fact a slight modification of the sample preparation method develop for the determination of some antagonist drugs [50], was used. By adding a small amount of 5% $ZnSO_4.7H_2O$ solution, NaOH and ethanol to the urine or plasma samples which including $2x10^{-6}$ M SIM and centrifuging the mixture, most of the interfering substances (mainly proteins) were simply removed and eliminated by precipitation. As can be extracted from Table 1, this SW-AdSV method (after appropriate dilution) allowed the determination of the complex ($5x10^{-5}$ M Cd^{+2} constant) in spiked urine and plasma samples with mean recoveries 87% ±1.23 and 84% ±1.41, respectively.

Table 1. Analytical result for SIM-Cd(II) complex recovery from biological fluids.

	Spiked Urine	Spiked plasma
	% Drug Recovery	% Drug Recovery
Added SIM 2.0×10^{-6} mol l^{-1}	85 88 87 88	83 84 85 85
Mean	87	85
Standard Deviation	±1.23	±1.41

4. Conclusion

The present study describes for the first time, the development and validation of a sensitive voltammetric method for determination of simvastatin in human urine and plasma by complex formation with Cd (II). The proposed voltammetric technique has the advantages of being simpler, faster, more selective and more cost-effective than the reference methods. The SW-AdSV method are rapid, requiring about 5 min to run sample, and involve no sample preparation other than dissolving, diluting and transferring an aliquot of biological samples to the supporting electrolyte. The possibility of monitoring of the compound in human urine and plasma makes the voltammetric method useful for pharmacokinetic and pharmacodynamic purposes.

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