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A Novel Method for Determination of Folic Acid by Fast Continuous Cyclic Voltammetry in Pharmaceutical Preparation

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Abstract- In this work, for the first time a fast continuous cyclic voltammetry was used as a highly sensitive detection method for the monitoring of Folic acid in a flow-injection system. It was used for analysis of Folic acid in pure form as well as in its pharmaceutical formulations. The best performance for sensitive monitoring of Folic acid was obtained at pH 2.0, scan rate 70 Vs^{-1}, accumulation potential 300 mV and accumulation time 0.8 s. The most advantages of the proposed detection method are, including, the removal of oxygen from the test solution is not required any more, and detection limit of the method is in sub-nanomolar level. The potential waveform consisting of the potential steps for cleaning, accumulation and potential ramp was continuously applied on an Au disk microelectrode (with a 12.5 μm in radius). The detection limit of the method for Folic acid was 1.45pg/ml. The relative standard deviation of the method at 2.0×10^{-7} M was 2.1 % for 10 runs.

Keywords- Folic Acid, Fast Fourier Ttransformation, Microelectrode, Continuous Cyclic Voltammetry, Flow Injection Analysis

1. INTRODUCTION

Folates and its derivatives occur as polyglutamates in nature. The multiplicity of forms and the generally low levels in foods makes quantitative analysis of folate a difficult task [1]. An LC-MS/MS method has been developed for the determination of main monoglutamate folates in spinach with folic acid as an internal standard [2].

A stable isotope dilution assay (SIDA) for the quantitation of A (2)-[1-(carboxy) ethyl] folic acid (CEF) also has been developed by using [H-2(4)] CEF as the internal standard. After sample cleanup by anion exchange chromatography, the three-dimensional specifity of liquid chromatography-tandem mass spectrometry enabled unequivocal determination of the nonenzymatic glycation product of folic acid (FA) [3]. Another sensitive procedure using capillary zone electrophoresis (CZE) to measure methotrexate, folinic acid and folic acid in human urine has been reported and validated [4]. In addition, specific flow-injection spectrofluorimetric method has been developed for the determination of folic acid in pharmaceuticals. Under optimum conditions the fluorescence intensity of oxidation product is proportional to the concentration of folic acid over the range 0.008-2.5 mg/ml. The detection limit is 0.0001 mg/ml [5]. Single-wall carbon nano-tubes were used to modify the surface of a glassy carbon electrode (GC) and applied in the determination of folic acid with voltammetry. The reduction peak current was found to be linearly related to folic acid concentration over the range of 1×10^{-8} to 1×10^{-4} mol L^{-1} with a detection limit of 1×10^{-9} mol L^{-1} after 5 min accumulation [6]

The voltammetric behavior of folic acid (FA) at a multi-walled carbon nanotube (MWNT) modified gold electrode has been investigated by cyclic voltammetry, chronoamperometry and chronocoulometry. Under the optimum conditions, the peak current changes linearly with FA concentration in the range from 2.0×10^{-8} M to 1.0×10^{-6} M. This method has been applied to the determination of FA in drug tablets, and the recovery is 93.9-96.9 % [7]

Our work introduces another online method for monitoring of folic acid in pharmaceuticals based on an electrochemical techniques and flow injection analysis with a very low detection limit and more sensitive method.

2. EXPERIMENTAL SECTION

2.1. Reagents

All solutions were prepared in double-distilled deionized water; using analytical grade reagents. The reagents used to prepare the running buffer or background electrolyte (BGE) for flow-injection analysis were obtained from Merck Chemicals. In all of the experiments, solutions were made up in the background electrolyte solution, and were used without

removal of dissolved oxygen. Folic acid tablets (Tolid Daru Co., IRAN), containing a label claim of 1 mg folic acid, that was purchased from a local pharmacy.

2.2. Background electrolyte

The running buffer or BGE was made by addition of 8.7 ml of phosphoric acid (85 % w/v) into a 1000 ml volumetric flask and dilution to a constant volume with distilled water. The pH was adjusted to 2 with sodium hydroxide and all solutions were freshly prepared and filtered using a Millipore filter (0.45 μm) each day.

2.3. Standards and Sample Solutions

2.3.1. Standard stock solutions

A standard stock solution of folic acid (100 μg/ml) was prepared in distilled water. This solution was freshly prepared each day.

2.3.2. Standard solutions for FIA

Aliquots of standard stock solution of folic acid were dispensed into 10 ml volumetric flasks and the flasks made up to volume with the running buffer to give final concentrations range of 4.5-485540 pg/ml.

2.4. Assay sample preparation

Twenty tablets were weighed, finely powdered and portions equivalent to 1 mg folic acid were transferred into 100 ml volumetric flask; 50 ml distilled deionized water was added, shaken thoroughly to dissolve, made up to volume and mixed well. Suitable aliquots of solution were filtered through a Millipore filter (0.45 μm). 1 ml of the filtered solution was added to a 100 ml volumetric flask and made up to volume with 0.05 M phosphoric acid to yield starting concentration of 100000 pg/ml.

2.5. Electrode preparation

Gold UMEs (with a 12.5 μm, in radius) were prepared by sealing metal micro-wires (Good fellow Metals Ltd., UK) into a soft glass capillary. The capillary was then cut perpendicular to its length to expose the wire. Electrical contacts were made using silver epoxy (Johnson Matthey Ltd., UK). Before each experiment the electrode surface was polished for 1 min using extra fine carborundum paper and then for 10 min with 0.3 μm alumina. Prior to being placed in the cell the electrode was washed with water. In all measurements, an Ag (s) | AgCl (s) | KCl (aq, 1 M) reference electrode was used. The auxiliary electrode was made of a Pt tube, 1 cm length and 0.5 mm in diameter.

Table 1. Influence of changes in experimental conditions on the performance of FIA system

Parameter	modification	Folic acid (% recovery)
pH	1.8 2 2.3 2.5	101.3 98.9 100.2 100.3
flow rate ml/min	2.8 3.0 3.2	102.0 100.2 99.9
Buffer composition (M)	0.04 0.05 0.06	98.5 99.4 100.6
Lab. Temperature (ºC)	20 25 30	100.45 99.8 101.3

2.6. Flow Injection Setup

The equipment for flow injection analysis included a 10 roller peristaltic pump (UltrateckLabs Co., Iran) and a four–way injection valve (Supelco Rheodyne Model 5020) with a 50 µl sample injection loop. Solutions were introduced into the sample loop by means of a plastic syringe. The electrochemical cell used in flow–injection analysis is shown in Fig. 1. The volume of the cell was 100 µl. In all experiments described in this paper, the flow rate of eluent solution was 3 ml/min.

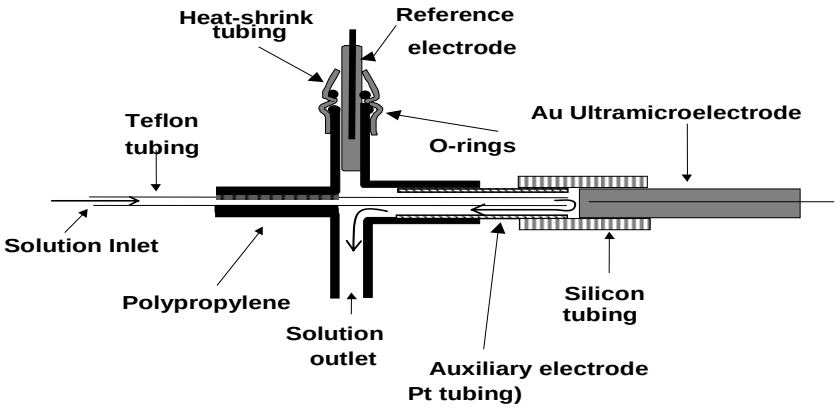

Fig. 1. Diagram of the electrochemical cell

2.7. Data Acquisition and Processing

All of the electrochemical experiments were done using a setup comprised of a PC PIV Pentium 900 MHz microcomputer, equipped with a data acquisition board (PCL-818HG, Advantech. Co.) and a custom made potentiostat. All data acquisition and data processing programs were developed in Delphi 6® program environment

In Fig. 2 the diagram of applied waveform potential during cyclic voltammetric measurements is shown. The potential waveform consists of three parts; a) Potential steps, E_{c1} and E_{c2} (which are used for oxidizing and reduction of the electrode surface, respectively), by which electrochemical cleaning of the electrode surface takes place, b) E_{acc}, where accumulation of analyte takes place, c) the final, part potential ramp, in which current measurements take place.

Signal Calculation in this method is established based on the integration of net current changes over the scanned potential range. It must be noted that in this case, the current changes (result of injected analyte) at the voltammograms can be caused by various processes, which take place at the electrode surface. Those processes include; a) oxidation and reduction of adsorbed analyte, and b) inhibition of oxidation and reduction of the electrode surface by the adsorbed analyte. Indeed, in order to see the influence of the adsorbed analyte on the oxidation and reductions peaks of the gold surface, the scan rate must be set at very high rates (e.g. >20 Vs^{-1}).

However, during the scan, some of the adsorbed analyte molecules are desorbed. Depending on the rate of those processes and scan rate, the amount of the desorption analyte molecule (during the scan) can be changed. The important point here is that part of the adsorbed analyte molecule still remaining on the electrode surface that can inhibited the red/ox process of the electrode surface. In this method, ΔQ is calculated based on the all current changes at the CVs [8-11]. However, the selectivity and sensitivity of the analyte response expressed in terms of ΔQ strongly depends on the selection of the integration limits. One of the important aspects of this method is application of a special digital filtration, which is applied during the measurement. In this method at the first, a CV of the electrode was recorded and then by applying FFT on the collected data, the existing high frequency noises were indicated. Finally, by using this information, the cutoff frequency of the analog filter was set at a certain value (where the noises were removed from the CV).

Since the crystal structure of a polycrystalline gold electrode, strongly depends on the condition of applied potential waveform [12] therefore various potential waveforms were examined in order to obtain a reproducible electrode surface (or a stable background signal). In fact, application of cyclic voltammetry for determination of electroactive compound mainly face to low stability of the background signal, due to changes occurring in the surface crystal structure during oxidation, and reduction of the electrode in each potential cycle. In this work, after examination of various potential wave forms, the best potential waveform for

obtains a stable background during the measurement was the waveform shown in Fig. 2. As mentioned above, in this work, the potential waveform was continuously applied during an experiment run where the collected data were filtered by FFT method before using them in the signal calculation. In Fig. 3 the CV of background (a) and after injection of analyte was presented. As can be seen the current in CVgram is changed by injection of sample which is related to adsorption of sample and inhibition of gold oxidation process.

The electrochemical oxidation process of gold surface started with electrosorption of hydroxyl ion, which at more positive potentials formation of gold oxide and undergoes structural rearrangement [13]. The surface oxidation can be initiated by adsorption of water molecule and then at more positive potential AuOH forms leading to the formation of a two-dimensional phase of gold oxide;

$$2\,Au + 3H_2O \longrightarrow Au_2O_3 + 6e + 6H^+ \qquad (1)$$

An example of recorded CVs is shown in Fig. 4(a, b). Fig. 4a shows a sequence of CVs recorded during the flow analysis for determination of the drug. The volume of the injection was of 50 μL of 1.0×10^{-6} M folic acid (in 0.05 M H_3PO_4) into the eluent solution containing 0.05 M H_3PO_4. The time axis of the graph represents the time of the flow injection experiment. In the absence of folic acid, the shape of the CV curves is typical for a polycrystalline gold electrode in acidic media [14]. Fig.4b shows the absolute current changes in the CVs curves after subtracting the average background 4 CVs (in absent of the analyte). Actually, The Fig. 4b is the differentiated form of Fig. 4a. It means that the determined current is subtracted of some CVs that recorded at the beginning of the experiment run. The region of gold oxidation is about 1000 mV and the reduction is about 400 mV when an analyte is adsorbed on the surface of electrode (we can use some samples by $K_{ads} > K_{ads}\ H_2O$) so formation of Au_2O_3 is reduced and the 3 electron reaction is reduced, so the change in current will be happen. The CV gram of gold electrode in absence of FA is presented in Fig. 3 as suggested. And then this system is a continuous system that it means many CVs have saved during every run and there is other dimension (time). As can be seen, this way of presentation of the electrode response gives more details about the effect of adsorbed ion on currents of the CV. The curves show that current changes mainly take place at the potential regions of the oxidation and reduction of gold. When the electrode-solution interface is exposed to folic acid, which can adsorbed on the electrode, the oxide formation process becomes strongly inhibited. In fact, the inhibition of the surface process causes significant change in the currents at the potential region, and as a consequence the profound changes in the shape of CVs take place. Universality of the detector in this mode is very advantageous for chromatographic analysis, where a mixture of compounds presents in sample. [15-19]

It must be noted that, theoretically, in this method, the analyte response can be affected by the thermodynamic and kinetic parameters of adsorption, the rate of mass transport and electrochemical behavior of the adsorbed species. The free energy and the rate of adsorption

depend on the electrode potential, the electrode material, and to some extent, on the choice of the concentration and type of supporting electrolyte. By taking points into consideration, in order to achieve maximum performance of the detector, the effect of experimental parameters (such as; pH of the supporting electrolyte, potential and time of the accumulation and potential scan rate) must be examined and optimized.

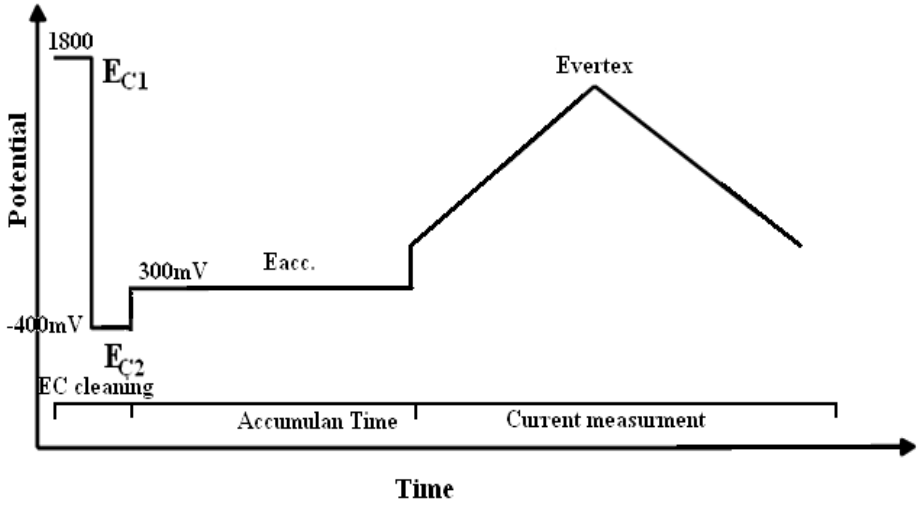

Fig. 2. Diagram of the applied potential waveform

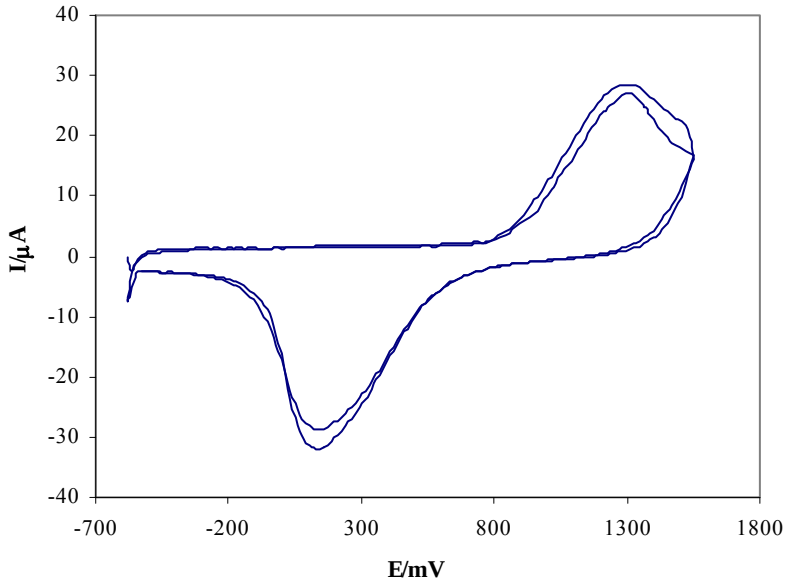

Fig. 3. The CV gram of electrode in the eluent 0.05 M H_3PO_4 (pH=2) and the flow rate was 3 mL min^{-1}, sweep rate was 70 Vs^{-1} (a) in the absence and (b) in the presence of analyte (1.0×10^{-6}M)

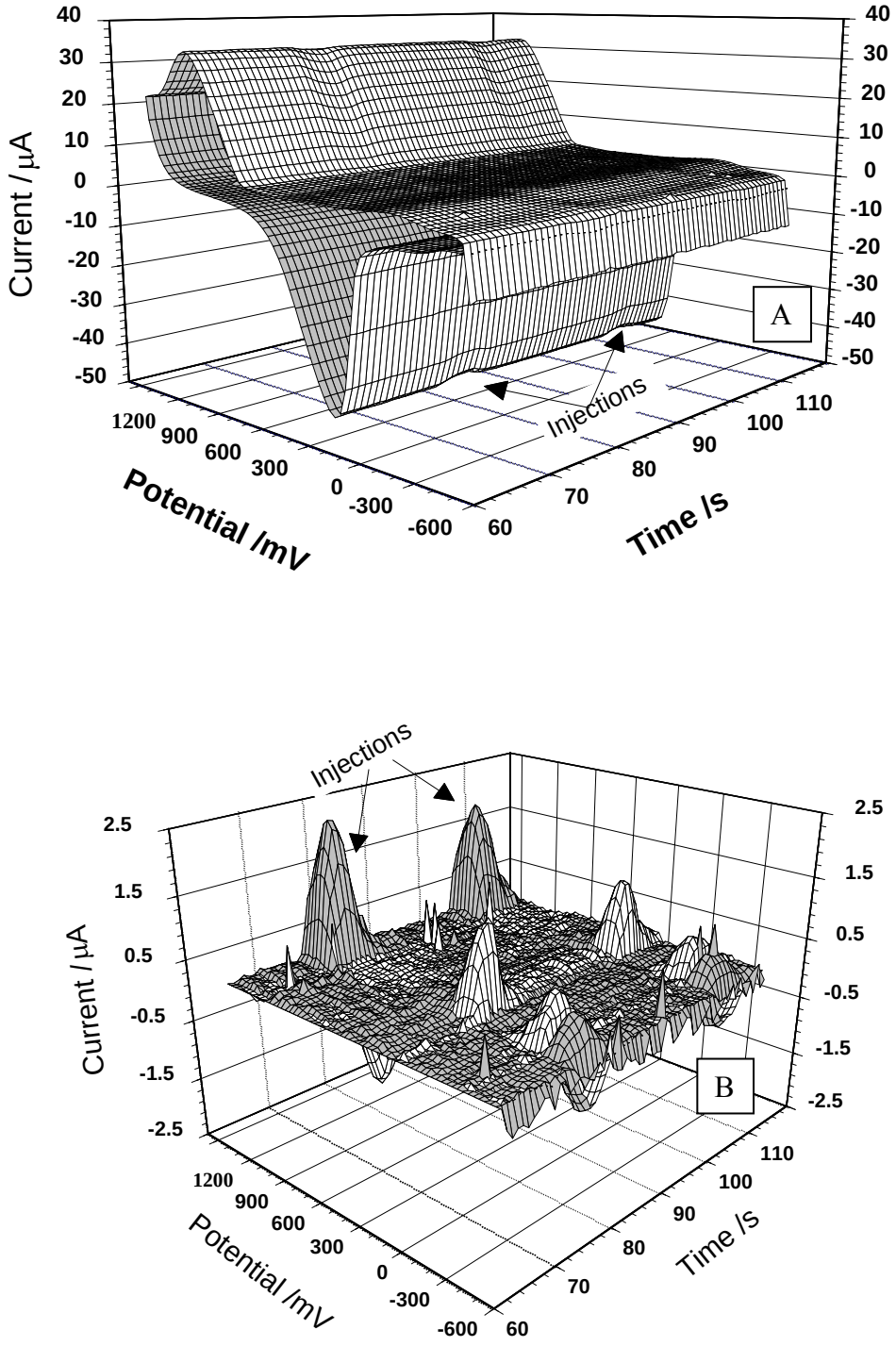

Fig. 4(a). Cyclic voltammograms at Au microelectrode recorded during the flow injection of 50 µL of 1.0×10^{-6} M of folic acid at optimum conditions. The eluent was 0.05 M H_3PO_4 and the flow rate was 3 mL min^{-1} b). Curves result from subtracting an average CV (in the absence of analyte) from test of the CVs in (a)

3. RESULTS AND DISCUSSIONS

3.1. Optimizing the Experimental Parameters

The effect of eluent pH on performance of the detector was examined the results are shown in Fig. 5 As shown, the highest current was obtained at pH=2. So this pH is selected for determination of folic acid.

Also, in order to investigate the influence of scan rates and the eluent flow rate on the sensitivity of the detector response, solutions having a concentration of 1.0×10^{-10} M folic acid were injected. At different scan rates (from 20 to 220 Vs^{-1}) and the eluent flow, the responses of the detector to the injected sample were recorded. The results are presented in Fig.6. As it is clear from the Fig.6 the detector exhibits the maximum sensitivity at 70 Vs^{-1} of scan rate and 3 ml/min of the flow rate. The effects of the sweep rate on the detection performance can be taken into consideration from three different aspects: first, speed in data acquisition, second, kinetic factors of adsorption of the folic acid, and finally the flow rate of the eluent which controls the time window of the solution zone in the detector. The main reason for application of high scan rates, is prevention from desorption of the adsorbed folic acid during the potential scanning, (because under this condition, the inhibition outcome of the adsorbed folic acid on the oxidation process can take place).Some other kind of similar structure adsorption were investigated in this manner [20-23].

Indeed, the use of this detection method in conjunction with fast separation techniques such as capillary electrophoresis also requires the employment of high scan rates. From this point of view, checking how the sensitivity of the method is affected by the sweep rate is necessary. To detect the amount of the adsorbed analyte on the electrode surface, high sweep rates must be employed, so that the potential scanning step is short in comparison with the accumulation period. Notably, when the accumulation of folic acid occurs at a potential that is very larger or smaller than E_i, this is very significant in this detection method. However, sensitivity of the detection system mainly depends on the potential sweep rate mainly due to kinetic factors in adsorption, and instrumental limitations.

Due to this fact that any changes in the parameters related to adsorption process shows a strong dependence upon the applied potential and the time and the potential of accumulation strongly affect the sensitivity of the measurement. Therefore, the influence of the accumulation potential and time on the response of the method for the injection of a solution of 1.0×10^{-10} M folic acid, in 0.05 M H_3PO_4, was studied. Fig. 7 shows the detector response over the accumulation potential ranges -700 to 700 mV and accumulation time range from 0.05 s to 1.0 s. Based the Fig accumulation potential 300 mV at time 800 ms was chosen as the optimum condition. Because, the surface of the electrode becomes saturated with the folic acid within 800 ms time window.

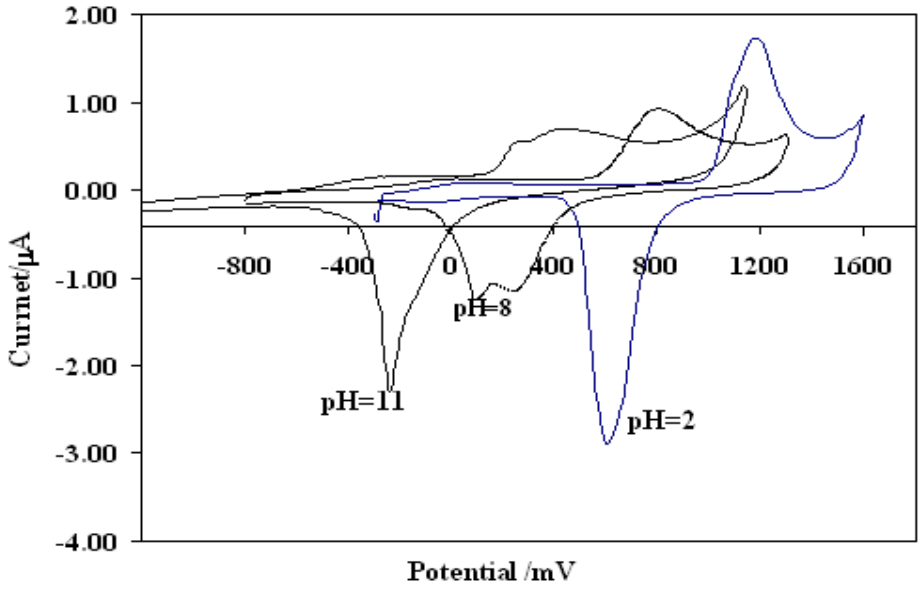

Fig. 5. Effect of pH on the response of electrode. The eluent was 0.05 M H_3PO_4 and the flow rate was 3 mL min^{-1} and sweep rate 70 Vs^{-1} in 1.0×10^{-7} M of Folic acid

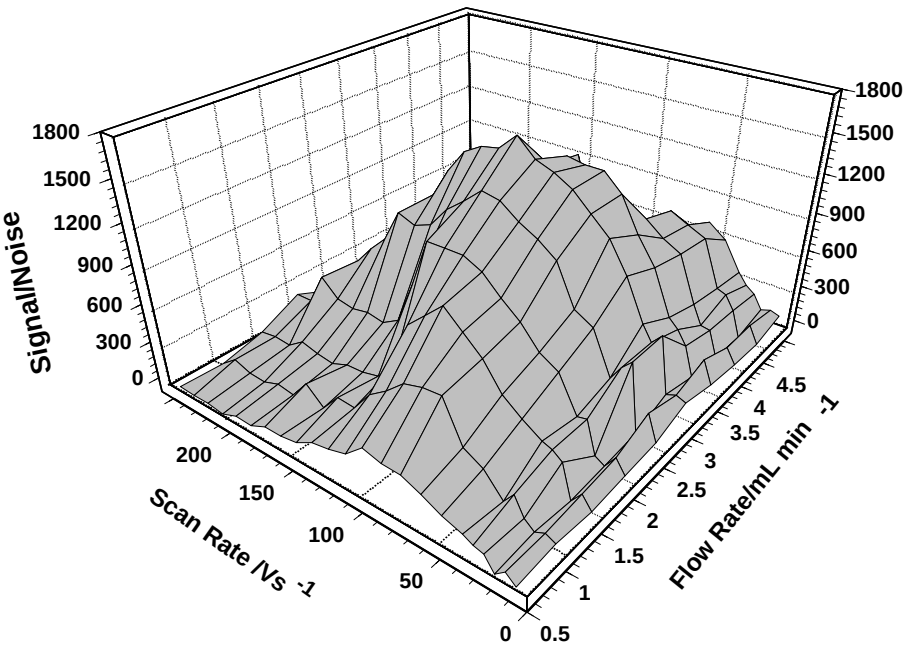

Fig. 6. Effect of the sweep rate on the response of the Au microelectrode to injections of 1.0×10^{-10} M folic acid in 0.05 M H_3PO_4

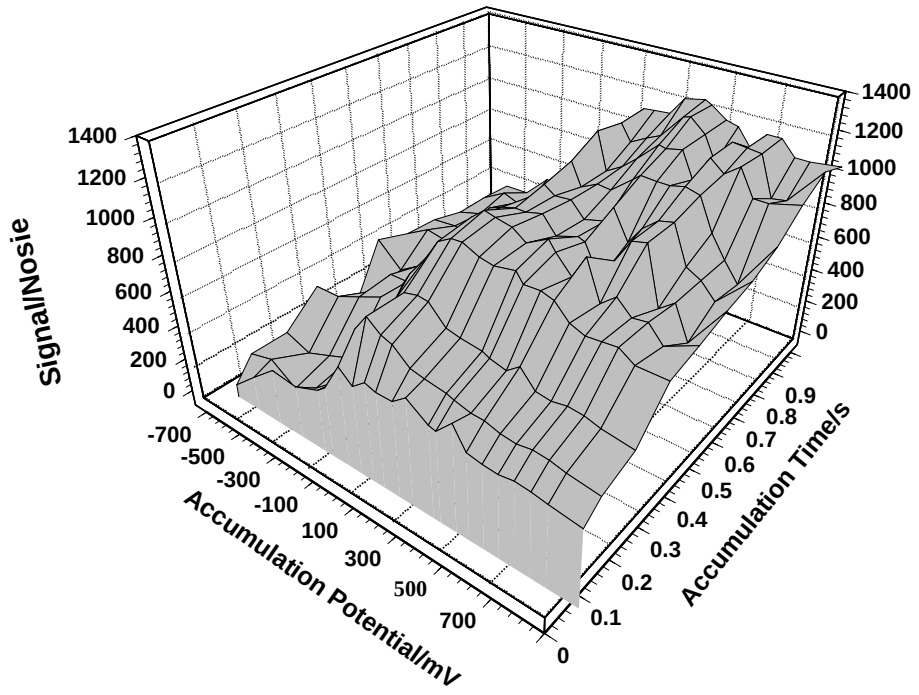

Fig. 7. Effect of accumulation potential and Effect of accumulation time on the electrode response to injections of 1.0×10^{-10} M folic acid in 0.05 M H_3PO_4

3.2. Validation

The method was validated with respect of linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, ruggedness/robustness, recovery and selectivity [24-26].

3.3. Linearity

The Linearity was evaluated by linear regression analysis, which calculated by the least square regression method [27, 28]. The calibration curves constructed for folic acid were linear over the concentration range of 4.5–485540 pg/ml. Peak areas of folic acid were plotted versus its concentration and linear regression analysis performed on the resultant curve. A correlation coefficient of R=0.996 with % R.S.D. values ranging from 0.15–3.75 % across the concentration range studied were obtained following linear regression analysis. Typically, the regression equation for the calibration curve was found to be Y=2.3909 X+2.8767. Fig. 8 shows the calibration graph that obtained for the monitoring of folic acid in a 0.05 M H_3PO_4.

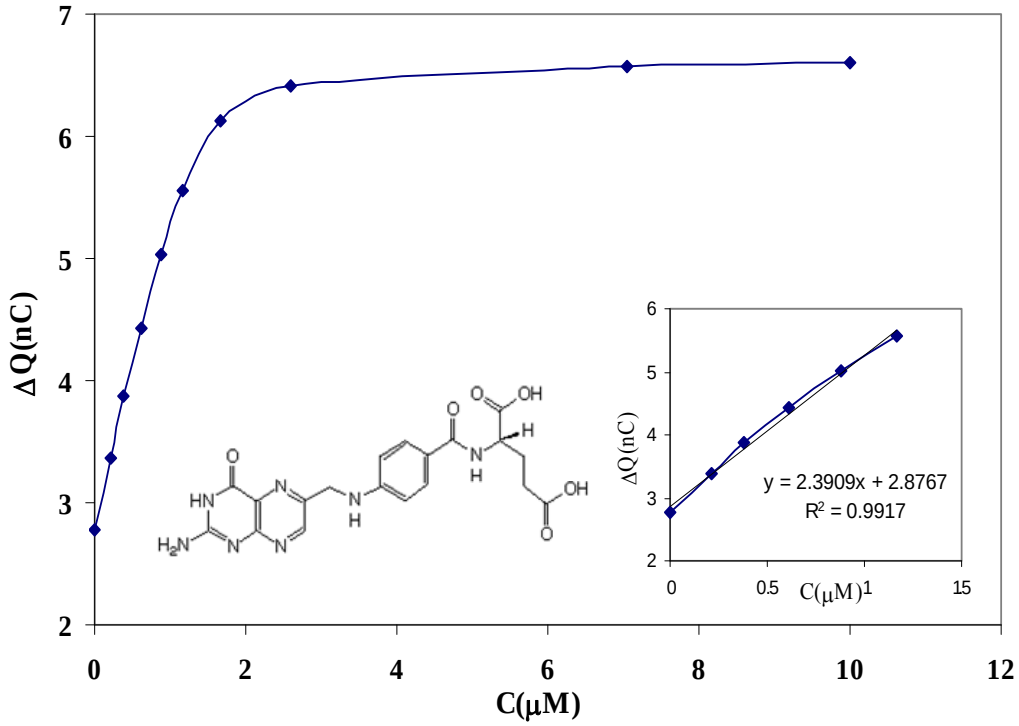

Fig. 8. Calibration curves obtained for folic acid on the Au microelectrode in 0.05 M H_3PO_4

3.4. LOQ and LOD

The LOQ and LOD were determined based on a signal-to-noise ratios and were based on analytical responses of 10 and 3 times the background noise, respectively [29]. The LOQ was found to be 4.5 pg/ml with a resultant %R.S.D. of 0.2% (n=5). The LOD was found to be 1.45 pg/ml.

3.5. Precision

Precision of the assay was investigated with respect to both repeatability and reproducibility. Repeatability was investigated by injecting nine replicate samples of each of the 4.5, 1000 and 485540 pg/ml standards where the mean concentrations were found to be 4.6, 1004 and 484045 with associated % R.S.D.'s of 3.3, 1.34 and 0.32, respectively. Inter-day precision was assessed by injecting the same three concentrations over 3 consecutive days, resulting in mean concentrations of folic acid of 4.57 , 1012 and 484011 pg/ml and associated % R.S.D. of 3.43 , 3.59 and 2.1%, respectively.

3.6. Ruggedness

The ruggedness of the method was assessed by comparison of the intra- and inter-day assay results for folic acid undertaken by two analysts. The % R.S.D. values for intra - and

inter – day assays of folic acid in the cited formulations performed in the same laboratory by the two analysts did not exceed 3.9 %, thus indicating the ruggedness of the method. Also the robustness of the method was investigated under a variety of conditions such as small changes in the pH of eluent, in the flow rate, in the buffer composition and in the laboratory temperature [30]. As can be seen in Table 2, the percent recoveries of folic acid were good under most conditions and did not show a significant change when the critical parameters were modified.

Table 2. Comparison between the Detection Limits of the Proposed Method with the other reported methods

No.	Method	LOD	Ref. No.
1	performance liquid-chromatography	50 ng/ml	[5]
2	performance liquid-chromatography	2000 ng/kg	[32]
3	capillary electrophoresis	3×10^{7}pg/ml	[33]
4	chromatography-tandem mass spectrometry	50 ng/ml	[34]
5	FFTCV	1.45 pg/ml	This work

3.7. Accuracy

Accuracy of the assay was determined by interpolation of replicate (n = 6) peak areas of three concentartion (4.5, 1000 and 485540 pg/ml) from a calibration curve prepared as previously described. In each case, the percent relevant error and accuracy was calculated. The resultant concentrations were 4.65±0.16 pg/ml, 982±13.7pg/ml and 484005±1210 pg/ml with percent relevant errors of 3.5, 1.3 and 0.31%, respectively. These concentrations were also evaluated by standard method (HPLC-UV) [31] and the results are 4.60±0.10 pg/ml, 978±14.5pg/ml and 484505±300 pg/ml.

3.8. Recovery

A known amount of folic acid standard powder was added to samples of tablets, which was then extracted, diluted and analyzed. The final nominal concentration of folic acid was found to be 109985pg/ml. The assay was repeated (n=9) over 3 consecutive days to obtain intermediate precision data. The resultant %R.S.D. for this study was found to be 1.43% with a corresponding percentage recovery value of 99.98 %.

3.9. Selectivity

The selectivity of the method was checked by monitoring standard solutions of folic acid in the presence of formulation components. The responses were not different from that obtained in the calibration. Hence, the determination of folic acid in this formulation is considered to be free from due to formulation components.

3.10. Assay of tablets

The method developed in the present study was applied for the determination of Folic acid in tablets from the Iranian market. The results showed a percent recovery of 100.2% and a R.S.D. of 1.6%. The result of standard method (HPLC-UV) [31] for determination of Folic acid is obtained for comparison by this method. The results were 99.8% recovery by RSD of 1.8%.

3.11. Comparison of the sensitivity of the method and other previously reported methods

Table 2, compares the detection limit of the proposed method with the other reported methods. As it is immediately obvious, the sensitivity of the method is superior to all previously reported methods. The data in Table 2 reveals that the detection limit of the method is about 34000 times lower than the most sensitive reported method.

4. CONCLUSION

In this work it is demonstrated that the concentration of folic acid in flowing solution can be measured indirectly via monitoring the current changes at oxidation and reduction of the electrode surface. In this method (adsorptive fast Fourier transform coulometric (AFFTC)) the S/N ratio is enhanced by using of fast Fourier transform of the analyte and signal integration. Also, for improving the sensitivity, the method take advantage of adsorption of the analyte on the Au microelectrode and the influence of adsorbed possible impurity in the eluent was removed by background subtraction. AFFTC can be considered as a new sensitive, accurate and fast method for determination of similar drugs, with ability of adsorption gold surface, in chromatographic systems, such as HPLC and CE. Finally, such detection limit (in nanomolar level), make the method suitable for bio-analysis. For instance, this method was applied for determination of folic acid in its tablet form (1.00±0.02 mg)

And had good agreement with the reported values (1.0 mg, Tolid Daroo, Iran). However, in order to obtain better sensitivity for a specific drug, experimental parameters should be optimized.

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