Monitoring of Anti Cancer Drug Letrozole by Fast Fourier Transform Continuous Cyclic Voltammetry at Gold Microelectrode

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A continuous cyclic voltammetric study of letrozole at gold microelectrode was carried out. The drug in phosphate buffer (pH 2.0) is adsorbed at −200 mV, giving rise to change in the current of well-defined oxidation peak of gold in the flow injection system. The proposed detection method has some of advantages, the greatest of which are as follows: first, it is no more necessary to remove oxygen from the analyte solution and second, this is a very fast and appropriate technique for determination of the drug compound in a wide variety of chromatographic analysis methods. Signal-to-noise ratio has significantly increased by application of discrete Fast Fourier Transform (FFT) method, background subtraction and two-dimensional integration of the electrode response over a selected potential range and time window. Also in this work some parameters such as sweep rate, eluent pH, and accumulation time and potential were optimized. The linear concentration range was of 1.0×10^{-7}—1.0×10^{-10} mol/L (r=0.9975) with a limit of detection and quantitation 0.08 nmol/L and 0.15 nmol/L, respectively. The method has the requisite accuracy, sensitivity, precision and selectivity to assay letrozol in tablets. The influences of pH of eluent, accumulation potential, sweep rate, and accumulation time on the determination of the letrozol were considered.

Keywords letrozole, cyclic voltammetry, fast Fourier transform

Introduction

Letrozole, 4-[(4-cyanophenyl)-(1,2,4-triazol-1-yl)-methyl] benzonitrile, with commonly used brand name Femara (Figure 1), is a non-steroidal triazole derivative and one of the most potent aromatase inhibitors that has been introduced for the adjuvant treatment of hormonally-responsive breast cancer.[1] It has been approved for the first-line therapy for hormone-receptor positive, metastatic breast cancer in postmenopausal women.[2] It has potential for use both to prevent the conversion of androgenic steroids to estrogens and to prevent or diminish the side effects of androgenic steroid abuse.[2] Letrozole is capable of inhibiting aromatase 98%—99% and reducing serum concentrations of estrone and E2 beyond the limit of detection in patients.[1]

Some of techniques described above are not simple for direct application to a large scale routine analysis and require expensive instruments. In pharmaceutical, biomedical and food analysis, a tendency can be observed towards the development of miniaturized and fast methods to achieve a highly sensitive determination. Recently, the stripping voltammetric methods were used in determination of heavy metal ions and some organic compounds in flowing solutions with a parts-per-billion sensitivity range. Indeed, the application of such techniques needs fast analyte accumulation and fast potential sweeping, which is not appropriate for large electrodes.[3,4] UMEs (ultra microelectrodes), for instance, have been applied as sensors in various techniques such as flow injection analysis,[5,6] organic compound analysis.[7,8] Now, our work describes a new electrochemical method based on FIA and FFT cyclic voltammetry for determination of letrozole.

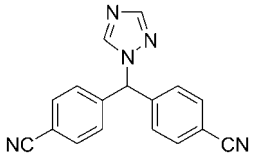

Figure 1 Schematic of letrozole structure.

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Experimental

Reagents and materials

Double-distilled deionized water was used for preparation of samples by using analytical grade reagents (Merck Chemicals). The reagents used for preparation of the running buffer or background electrolyte (BGE) solution for flow-injection analysis (0.05 mol/L H_3PO_4 and 1 mol/L NaOH used for adjusting pH of the eluent), were obtained from Merck Chemicals. Letrozol standard obtained from Novartis pharmaceuticals, (Dorval, Quebec, Canada). In all experiments, solutions were made up in the background electrolyte solution, and used without removal of dissolved oxygen.

Background electrolyte

The background electrolyte was made by addition of 8.7 mL of phosphoric acid (85%) 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.

Standards and sample solutions

Standard stock solutions A standard stock solution of letrozol (1 mg/mL) was prepared in the distilled water and ethanol in 1∶1 (*V*∶*V*). This solution was protected from light using foil and stored at 4 ℃.

Standard solutions for FIA Aliquots of standard stock solution of letrozole were dispensed into 10 mL volumetric flasks and the flasks made up to volume with the running buffer to give final concentrations range of 1.0×10^{-5}—1.0×10^{-10} mol/L.

Sample preparation of human urine and plasma

Plasma was obtained from Tehran University Hospital, Tehran, Iran and kept frozen until use after gentle thawing. Urine was also collected from healthy volunteers (males, around 30-years-old).

1 mL of untreated urine containing 10 μg/mL letrozole was placed into a 50 mL volumetric flask and diluted with water to the mark. A 1 mL of this solution was diluted with pH 2 buffer solution to 20 mL into a volumetric flask. Then 20 μL aliquot was injected into the FIA system.

For the determination of letrozole in plasma, 100 μL aqueous letrozol solutions (100 ng/mL) were added to 1 mL of untreated plasma. The mixture was vortexes for 30 s. In order to precipitate the plasma proteins, the plasma samples were treated with 20 μL perchloric acid $HClO_4$ 20%. After that, the mixture was vortexed for a further 30 s and then centrifuged at 6000 r/min for 5 min. Then 20 μL aliquot of the obtained supernatant was injected into the FIA system.

Electrode preparation

Gold UMEs (with a 25 μm in diameter) were prepared as described in our previously papers.[14-18] 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 mol/L) reference electrode was used. The auxiliary electrode was made of a Pt wire, 1 cm in length and 0.5 mm in diameter.

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 volume of the cell was 100 μL. In all experiments described in this paper, the flow rate of eluent solution was 0.5 mL/min.

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.

Results and discussion

In Figure 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_c, where accumulation of analyte takes place, (c) the final, part potential ramp, in which current measurements take place.

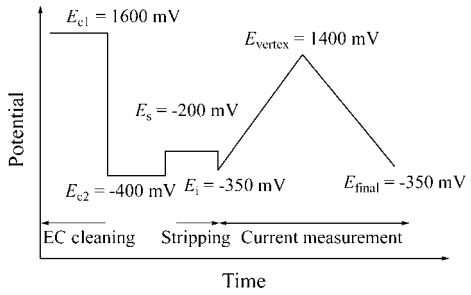

Figure 2 Diagram of the applied potential waveform.

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 V/s).

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.[9-24] The important point here is that part of the adsorbed analyte molecules still remain on the electrode surface that can inhibit the red/ox process of the electrode surface. In this method, ΔQ is calculated based on the all current changes at the CVs. However, the selectivity and sensitivity of the analyte response expressed in terms of ΔQ strongly depend 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,[4] 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 waveforms, the best potential waveform for obtaining a stable background during the measurement was the waveform shown in Figure 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.

The electrochemical oxidation process of gold surface started with electrosorption of hydroxyl ion, which occurs at more positive potentials and causes structural rearrangement.[14,15] 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:

$$2Au + 3H_2O \rightarrow Au_2O_3 + 6e^- + 6H^+ \quad (1)$$

An example of recorded CVs is shown in Figure 3 (a, b).

Figure 3a 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 3.0×10^{-6} mol/L letrozol (in 0.05 mol/L H_3PO_4) into the eluent solution containing 0.05 mol/L H_3PO_4. The time axis of the graph represents the time of the flow injection experiment. In the absence of letrozol, the shape of the CV curves is typical for a polycrystalline gold electrode in acidic media.[15] Figure 3b shows the absolute current changes in the CVs curves after subtracting the average background 4 CVs (in absent of the analyte). 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 letrozole, 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.

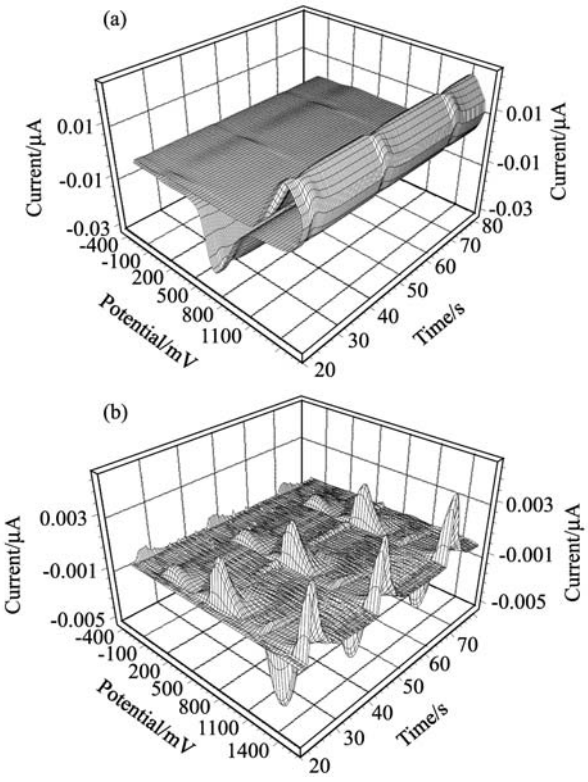

Figure 3 (a) Cyclic voltammograms at Au ultra-microelectrode recorded during the flow injection of 50 μL of 3.0×10^{-6} mol/L of letrozol at optimum conditions. The eluent was 0.05 mol/L H_3PO_4 and the flow rate was 0.5 mL/min. (b) Curves resulting from subtracting an average CV (in the absence of analyte) from test of the CVs in (a).

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 adsorp-

tion 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.[24-32]

Optimizing the experimental parameters

The effect of eluent pH on performance of the detector was examined and the results are shown in Table 1. As shown, the best ΔQ was obtained between pH 2—3. In addition, the results show that at pH values higher than 9 noises level in the baseline (ΔQ vs. Time), is higher up to 12% compared to acidic solution.

Table 1 pH effect on the microelectrode response

pH	2.1	4	6	8	10	12
ΔQ/μC	0.02	0.018	0.016	0.015	0.014	0.014

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 5.0×10^{-7} mol/L of letrozol were injected. At different scan rates (from 20 to 80 V/s) and the eluent flow, the responses of the detector to the injected sample were recorded. The results are presented in Figure 4. As it is clear from Figure 4, the detector exhibits the maximum sensitivity at 30 V/s of scan rate and 0.5 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 letrozole, 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 letrozole during the potential scanning. Under this condition, the inhibition outcome of the adsorbed letrozole on the oxidation process can take place.

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 letrozole 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.

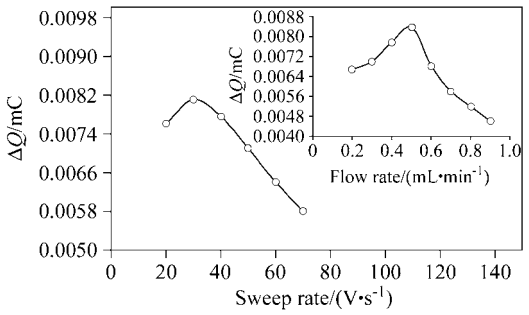

Figure 4 Effect of the sweep rate on the response of the Au microelectrode to injections of 5.0×10^{-7} mol/L letrozole in 0.05 mol/L H_3PO_4.

Due to this fact that any change 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 5.0×10^{-7} mol/L letrozol, in 0.05 mol/L H_3PO_4, was studied. Figure 5 shows the detector response over the accumulation potential ranges −700 to 800 mV and accumulation time range from 0.1 s to 1.0 s. Based on the figure accumulation potential −200 mV at time 400 ms was chosen as the optimum condition. The surface of the electrode becomes saturated with the letrozole within 200 s time window.

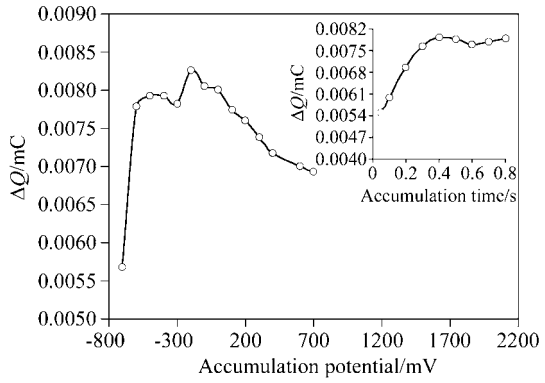

Figure 5 Effect of accumulation potential (a) and the effect of accumulation time (b) on the electrode response to injections of 5.0×10^{-7} mol/L letrozol in 0.05 mol/L H_3PO_4.

On the electrode, the accumulation of letrozol takes place during the accumulation step (assuming that an appropriate potential is selected). In fact, the difference in the time of saturation of the various compounds can be related to the existing differences in their kinetics of the electron transfer and mass transport. As mentioned above, the surface of the gold microelectrode is very small, and in a very short time the surface of the electrode can be saturated.

Validation

The investigation of validity was performed with respect of linearity, limit of detection (LOD), precision, accuracy, ruggedness/robustness, recovery and selectivity.[33-35]

Linearity

Linear regression analysis of least square method was used to evaluate the linearity.[36,37] The linear range of 0.2—0.0001 μmol/L was conspicuous in constructed calibration curve. Peak areas of letrozol were plotted versus its concentration and linear regression analysis performed on the resultant curve. A correlation coefficient of $R=0.9972$ with RSD values ranging from 0.18%—3.9% across the concentration range studied were obtained following linear regression analysis. Typically, the regression equation for the calibration curve was found to be $Y=0.00001x+0.0008$. Figure 6 shows the calibration graph that obtained for the monitoring of letrozole in a 0.05 mol/L H_3PO_4.

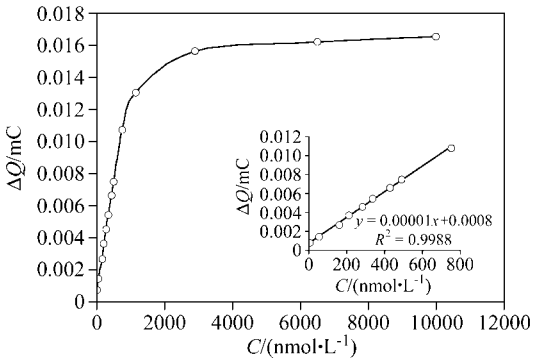

Figure 6 Calibration curves obtained for letrozole on the Au microelectrode in 0.05 mol/L H_3PO_4.

LOD

The lowest amount of the analyte that may be detected to produce a response is defined as LOD. Based on the calculation of standard deviation of the response (δ) and the slope (S) of the calibration curve at the levels approaching the limits according to equation $LOD=3.3(\delta/S)$,[38] the limit of detection that found to be 0.08 nmol/L, was approved.

Precision

Precision was investigated by injecting nine replicate samples of each of the 0.2, 0.08 and 0.008 μmol/L standards. The final mean concentrations were found to be 0.19, 0.07 and 0.0088 μmol/L with associated RSD's of 0.5%, 1.0% and 1.1%, respectively. The inter-day precision was assessed by injecting the same three concentrations for 3 consecutive days, resulting in mean Letrozole concentrations of 0.2, 0.078 and 0.008 μmol/L with associated RSD values of 0.5%, 0.7% and 1.0%, respectively.

Accuracy

Interpolating of replicate ($n=6$) peak areas of three accuracy standards (0.2, 0.08 and 0.008 μmol/L), the accuracy of the method was assessed by a calibration curve prepared as previously described. In each case, the percent relevant error and accuracy was calculated. The resultant concentrations were (0.2 ± 0.05), (0.08 ± 0.005) and (0.007 ± 0.0001) μmol/L with relevant error percentage of 0.65%, 0.9% and 0.85%, respectively.

Ruggedness

Comparing of the intra- and inter-day assay results for two letrozole analytes was used to check the ruggedness of the method. The RSD values for intra- and inter-day assays of letrozol in the cited formulations performed in the same laboratory by the two analysts did not exceed 4.5%. The robustness was also examined while the parameters values (the pH of the eluent, the flow rate, the buffer composition and the laboratory temperature) were being slightly changed.[39] According to Table 2, the letrozole recovery percentages were satisfactory in most cases, without presenting any important changes during the alteration of the critical parameters.

Table 2 Influence of the changes in the experimental conditions on the performance of the FIA system

Parameter	Modification	Letrozol recovery/%
pH	1.8	100.3
	2.0	101.1
	2.3	99.9
	3.0	99.8
Flow rate/(mL•min^{-1})	2.8	100.6
	3.0	101.3
	3.2	99.9
Buffer composition/(mol•L^{-1})	0.04	97.9
	0.05	101.6
	0.06	100.4
Lab. temperature/℃	20	101.3
	25	99.9
	30	100.8

Recovery

In order to perform the recovery test, letrozole standard powder at concentration of 0.5 ng/mL was added to samples of known amounts at 0.2, 0.08 and 0.008 μmol/L and then the voltammograms were recorded. The assay was repeated ($n=9$) over 3 consecutive days to obtain intermediate precision data. The resultant RSD for this study was found to be 0.9% with a corresponding percentage recovery value of 99.95%.

Selectivity

Standard solutions of letrozole, was exploited to determine the sensitivity of the method in the presence of formulation components. As expected, the responses were not different from that obtained in the calibration. We found that the formulation compounds have no interference to the determination of letrozole due to the well fixed optimized parameters.

Determination of letrozole in real samples

The voltammograms were recorded according to the above recommended procedure. The voltammograms of samples without letrozole do not show any signal that can interfere with the direct determination, so external calibration can be used. The result has been shown in Table 3. The major advantage of the method as applied to plasma and urine is that no prior extraction step is required.

Table 3 Application of the proposed method to the determination of letrozole in spiked human plasma and urine[a]

Added/(ng•mL^{-1})	Interpolated concentration/(ng•mL^{-1})	R.S.D./%	R.E./%
10 (plasma)	9.94±0.2	1.5	1.05
100 (urine)	101.2±0.5	1.0	1.6

[a] Data obtained from five replicates at each concentration. Interpolated concentration data expressed as mean±S.D.

Conclusion

In this work for the first time it is demonstrated that the concentration of letrozole in flowing solution can be measured in trace amounts indirectly via monitoring the current changes at oxidation and reduction of the electrode surface. In this method (FFTCV) the S/N ratio is enhanced by using of fast Fourier transform of the analyte and signal integration. Also, to improve the sensitivity, the method takes 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. FFTCV can be considered as a new sensitive, accurate and fast method for determination of similar drugs, with ability of adsorption on gold surface, in chromatographic systems, such as HPLC and capillary electrophoresis. However, in order to obtain better sensitivity for a specific drug, experimental parameters should be optimized. Finally, such detection limit (in nanomolar level), makes the method suitable for bio-analysis. For instants, this method was applied to determination of letrozole in its tablets and had good agreement with the reported values.

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