JSUT 33(2), 2007, p. 41-49

Non-Electroactive Recognition: Pico-Level Monitoring of Tamoxifen by its Sub-Second Adsorption at Au Microelectrode by Fast Fourier Transforms Continuous Cyclic Voltammetric Technique (FFTCCV)

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(*received: 11/12/2007 ; accepted: 14/1/2008*)

Abstract

In this work a novel method for the determination of tamoxifen in flow-injection systems has been developed. The fast Fourier transform continuous cyclic voltammetry (FFTCV) at gold microelectrode in flowing solution system was used for determination of tamoxifen in its pharmaceutical formulations. The developed technique is very simple, precise, accurate, time saving and economical, compared to all of the previously reported methods. The effects of various parameters on the sensitivity of the method were investigated. The best performance was obtained with the pH value of 2, scan rate value of 50V s^{-1}, accumulation potential of 0 mV and accumulation time of 0.5 s. The proposed method has some advantages over other reported methods such as, no need for the removal of oxygen from the test solution, a picomolar detection limit, and finally the method is fast enough for the determination of any such compound, in a wide variety of chromatographic methods. The method was linear over the concentration range of 1875-78000 pg/ml (r = 0.9980) with a limit of detection and quantitation 37.1 and 200 pg/ml, respectively. We used this method to determination of tamoxifen in tablets.

Keywords: Tamoxifen, Ultra-amicroelectrode, Fast Fourier Transform Cyclic Voltammetry, Flow injection.

Introduction

More than 60% of human breast tumors are estrogen receptor (ER)-positive (ERþ) and depend on estrogens for growth. The anti-estrogen tamoxifen (TAM) is currently the first-line medicine for treatment of ERþ breast cancer in both pre- and postmenopausal women (Fisher *et al.* 1996).

Tamoxifen has also been demonstrated its efficacy on the prevention of ERþ breast tumors **(**Fisher *et al.* 1998). Tamoxifen, Figure 6, The trans isomer of (Z)-2-[p-(1,2-diphenyl-butenyl)phenoxy]-N,N-dimethyletylamine (tamoxifen**)**which itself is a pro drug, having very little affinity for its target protein, the estrogen receptor. It must first be metabolized in the liver by the cytochrome P450 isoform CYP2D6 into the active metabolites 4-hydroxytamoxifen and des-N-methyl-4-hydroxytamoxifen (endoxifen) (Desta *et al.* 2004). These active metabolites compete with estrogen in the body for binding to the estrogen receptor. In breast tissue, 4-hydroxytamoxifen acts as an antagonist so that transcription of estrogen-responsive genes is inhibited (Wang *et al.* 2004). Even though it is an antagonist in breast tissue it acts as partial agonist on the endometrium and has been linked to endometrial cancer in some women. Therefore endometrial changes, including cancer, are among tamoxifen's side effects (Grilli 2006).

Tamoxifen is an orally antiestrogen drug most commonly used in low dosages (20 mg daily). High-dose tamoxifen chemotherapy also may be used for the treatment of brain malignancies. Base of these reasons determination of this drug and its matabolits is so important. There was some reports on determination of that by using Capillary gas chromatographic in the presence of a number of antidepressants in urine (Rodriguez *et al.* 2003) and Cpillary zone electrophoresis (Flores *et al.* 2004), but this methods are very expensive and time consuming. The method which introduced in this paper is very sensitive, inexpensive and fast for detection of tamoxifen.

This techniques has been further stimulated by the advent of UMEs. Some of their special characteristics are their steady state currents, their higher sensitivity due to the increased mass transport and their ability to be used in solutions with high resistance. For instance, UMEs have been

applied as sensors in various techniques such as flow injection analysis (Li SFY, 1992**,** Dimitrakopoulos *et al.* 1996), cardiovascular monitoring and organic compounds analysis (Dimitrakopoulos *et al.* 1996, Hintsche *et al.* 1994, Sreenivas *et al.* 1996) This study aims to introduce a novel method for the fast determination of losartan ultra trace amounts in its pharmaceutical preparation.

Experimental parts

Reagents. All of the solutions were prepared in double-distilled deionized water, using analytical grade reagents. The reagents used to prepare the eluent solution 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. Tamoxifen standard powder was a gift from quality control of drug and food (Teharan, Iran) Nolvadex tablets (Netherlands) containing 10 mg tamoxifen was purchased from a local pharmacy.

Background electrolyte (BGE). The running buffer or BGE was made by addition of 8.7ml of phosphoric acid (85% w/v) into a 1000ml volumetric flask and dilution to a constant volume with distilled water. The pH was adjusted to 2.3 with sodium hydroxide and all solutions were freshly prepared and filtered using a Millipore filter (0.45μm) each day.

Standards and Sample Solutions.

Standard stock solutions. A standard stock solution of tamoxifen (1mg/ml) was prepared in water. This solution was freshly prepared each day.

Standard solutions for FIA. Aliquots of standard stock solution of tamoxifen were dispensed into 10 ml volumetric flasks and the flasks made up to volume with the running buffer to give final concentrations range of 1857- 78000pg/ml.

Assay of Tablets. The samples were prepared by weighing twenty tablets. Afterwards, the tablets were finely powdered and tamoxifen portions of 10 mg were transferred into 50 ml volumetric flask; 50 ml of distilled water were added, shaken completely to dissolve and mixed well.

Suitable aliquots of this solution were filtered through a Millipore filter (0.45 μm) and 100 μl of the filtered solution were diluted with distilled water in a 100 ml volumetric flask. Finally, 1 ml of the resulting solution was added to a 100 ml volumetric flask and filled up with 0.05 M phosphoric acid, reaching an initial concentration of 2000 pg/ml.

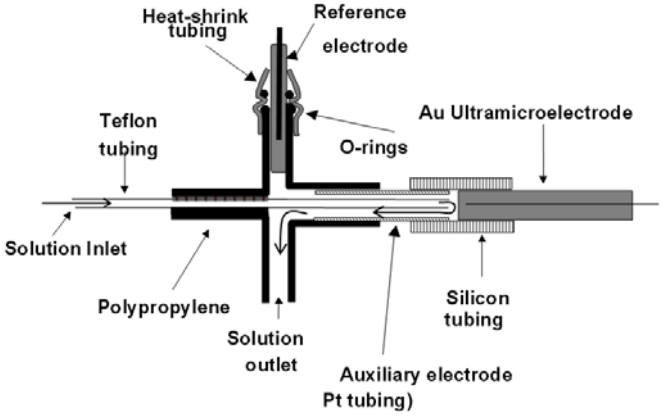
Sample preparation of human urine and plasma. Plasma was obtained from Tehran University Hospital, Tehran, Iran and kept frozen until use after gentle thawing. Urine was also collected from healthy volunteers (males, around 30-years-old). One ml of untreated urine containing 50 ng/mL tamoxifen was placed into a 5 mL volumetric flask and diluted with water to the mark. A 1 mL of this solution was diluted with pH 2 buffer solution to 5 mL into a volumetric flask. Then 20μL aliquot was injected into the FIA system.

For the determination of tamoxifen in plasma, 100 μL aqueous tamoxifen solutions (50ng/mL) were added to 100 μL of untreated plasma. The mixture was vortexes for 30s. 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 30s and then centrifuged at 6000 rpm for 5 min. Then 20μL aliquot of the obtained supernatant was injected into the FIA system.

Electrode preparation. Gold UMEs (with a radius of 12.5 μm) 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 Mat they Ltd., UK). Before each experiment the electrode surface was polished for 1 minute using extra fine carborundum paper and then for 10 minutes 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, 1M) reference electrode was used. The auxiliary electrode was made of a Pt wire, 1cm in length and 0.5 mm in diameter.

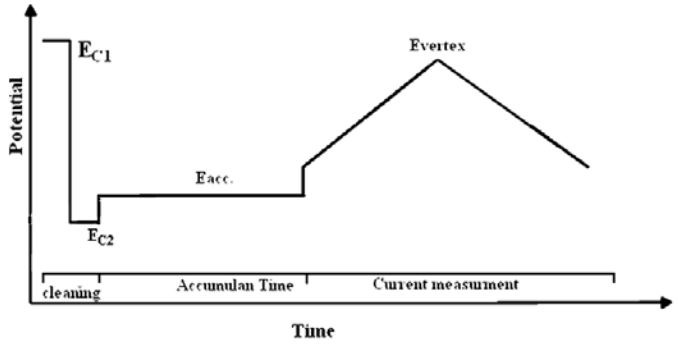
Flow injection setup. The equipment for flow injection analysis included a six roller peristaltic pump (LKB 2115 Miltiperpex Co.) and a fourways injection valve (Supelco Rheodyne Model 5020) with a 50 μl sample injection loop. Solutions were introduced into the sample loop by means of a plastic syringe. The electrochemical cell used in flowinjection analysis is shown in Figure 1. The

volume of the cell was 100 µl. In all experiments described in this paper, the flow rate of eluent solution was 100µl/s.



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 Figure 2, the diagram of applied waveform potential during cyclic voltammetric measurements is shown. The potential waveform consists of three parts; a) Potential steps, Ec1 and Ec2 (which are used for oxidizing and reduction of the electrode surface, respectively), by which electrochemical cleaning of the electrode surface takes place, b) Ec, 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 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. The important point here is that part of the adsorbed analyte molecule still remaining 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. (Norouzi *et al.* 2005a&b, Ganjali *et al.* 2005a&b, Norouzi *et al.* 2006, Wightman & Wipf 1989) 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, (Lipkowski & Stolberg 1992) 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 Figure 2. As mentioned above, in this work, the potential

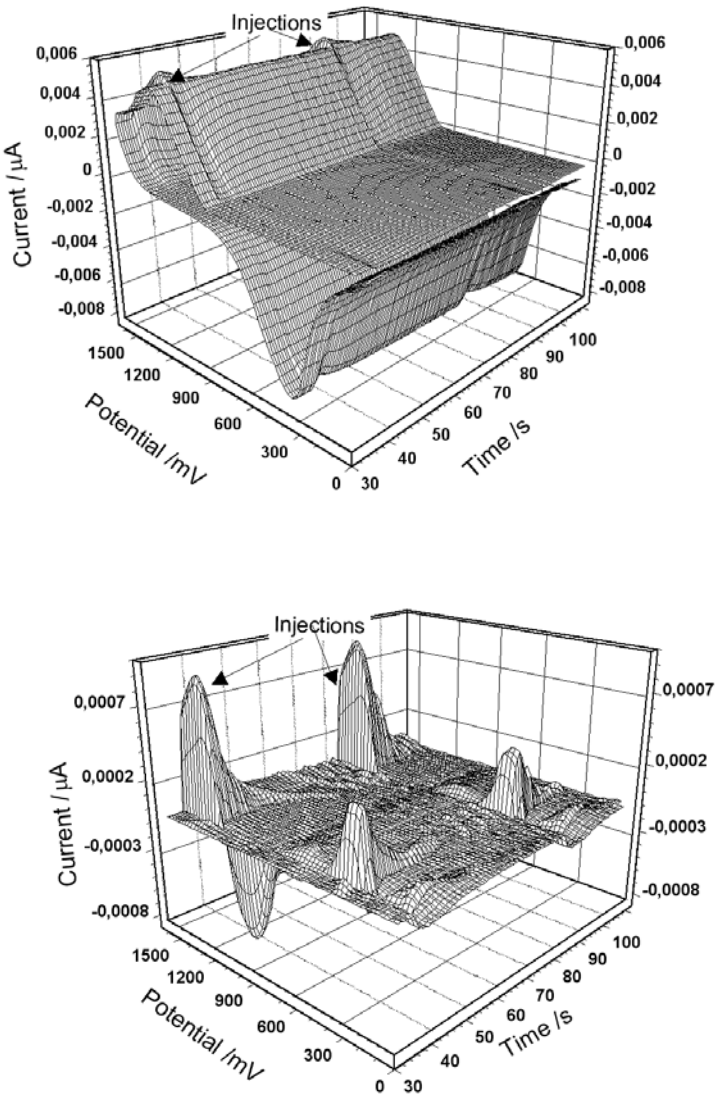
waveform was continuously applied during an experiment run where the collected data were filtered by FFT method before using them in the signal calculation.

The electrochemical oxidation process of gold surface started with electrosorption of hydroxyl ion, which at more positive potentials formation of gold oxide and undergoes structural rearrangement (Bockris *et al.* 1980). 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 oxid,

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

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 5.0×10^{-6} M tamoxifen (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 tamoxifen, the shape of the CV curves is typical for a polycrystalline gold electrode in acidic media.[32] Figures 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 tamoxifen, 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.

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 ccumulation and potential scan rate) must be examined and optimized (Norouzi *et al.* 2007a~m, Nabi Bidhendi *et al.* 2007).



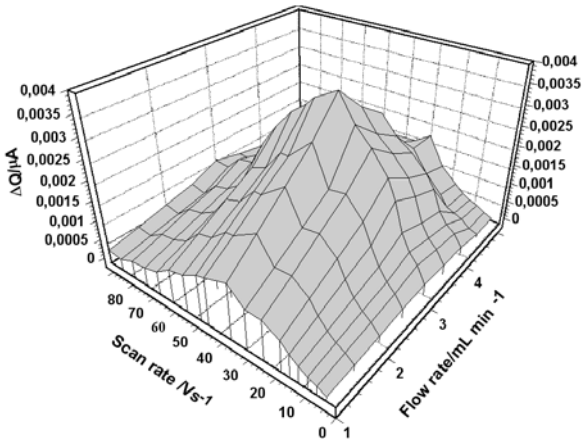
Results and Discussion.

Optimizing the experimental parameters. The effect of eluent pH on performance of the detector was examined the results are shown in Table 1. As shown, the best S/N ratio was obtained between pH 2-3. In addition, the results shows 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. Effect of pH on the response of microelectrode.

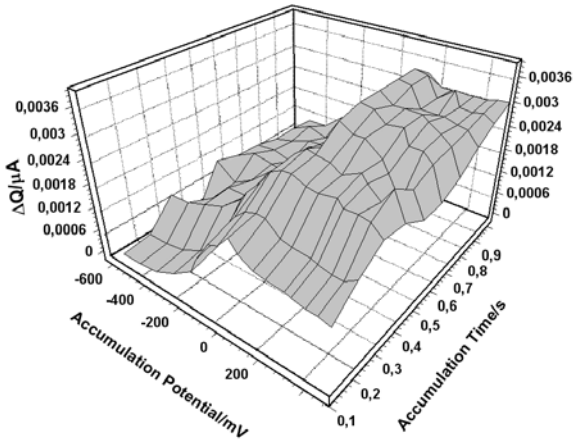
pH	2.1	4	6	8	10	12
S/N	120	105	90	84	76	79

For investigation on 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} M tamoxifen were injected. At different scan rates (from 5 to 90 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 the Figure 4, the detector exhibits the maximum sensitivity at 50 V/s of scan rate and 3mL/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 tamoxifen, 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 tamoxifen during the potential scanning, (because under this condition, the inhibition outcome of the adsorbed tamoxifen 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 tamoxifen occurs at a potential that is very larger or smaller than Ei, 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 5.0×10^{-7} M tamoxifen, in 0.05 M H_3PO_4, was studied. Figure 5. shows the detector response over the accumulation potential ranges 400 to -600 mV and accumulation time range from 0.05s to 0.9s. Based the figure accumulation potential 0mV at time 500 ms was chosen as the optimum condition. Because, the surface of the electrode becomes saturated with the tamoxifen within 400 ms time window.



On the electrode, the accumulation of tamoxifen 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 ultramicroelectrode is very small, and in a very short time the surface of the electrode can be saturated.

After optimization the parameters, the calibration graph was prepared by injection of tamoxifen in concentration between 1857-78000 pg/ml that it is presented in fig. 6, and as it's clear, the electrode will be saturated in high concentrations and and the response of electrode is independent of concentration.

Validation. The method was validated with respect to parameters including linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, ruggedness/robustness, recovery and selectivity.

Linearity. The Linearity was evaluated by linear regression analysis, which calculated by the least square regression method (Mohammadi *et al.* 2005 &2006& 2007a,b, Nabi Bidhendi *et al.* 2007). The calibration curves constructed for tamoxifen were linear over the concentration range of 1857 – 78000 pg/ml. Peak areas of tamoxifen were plotted versus its concentration and linear regression analysis performed on the resultant curve. A correlation coefficient of R=0.9975 with %R.S.D. values ranging from 0.25 – 3.70 % across the concentration range studied were obtained following linear regression analysis. Typically, the regression equation for the calibration curve was found to be Y = 0.0042X+0.0002. Figure 6. shows the calibration graph that obtained for the monitoring of tamoxifen in a 0.05 M H_3PO_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 (Internatioal Conference on Harmonisation, ICH, Geneva 1996). The LOQ was found to be 200 pg/ml with a resultant %R.S.D. of 0.26% (n=5). The LOD was found to be 37.1 pg/ml.

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 1920, 5000 and 78000 pg/ml standards where the mean concentrations were found to be 1900, 5095 and 76900 pg/ml with associated %R.S.D.'s of 3.45, 1.45 and 0.24, respectively. Inter-day precision was assessed by injecting the same three concentrations over 3 consecutive days, resulting in mean concentrations of tamoxifen of 1950, 5080 and 7700 pg/ml and associated %R.S.D. of 3.25, 3.70 and 2.1%, respectively.

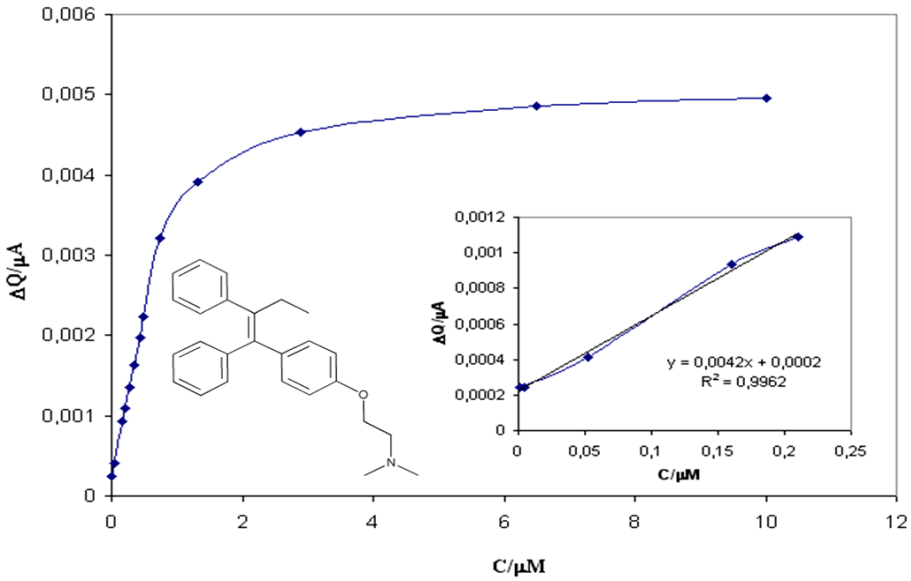
Accuracy. Accuracy of the assay was determined by interpolation of replicate (n = 6) peak areas of three accuracy standards (1900, 5000 and 77000 pg/ml) from a calibration curve prepared as previously described. In each case, the percent relevant error and accuracy was calculated. The resultant concentrations were 1870.1 ± 50 pg/ml, 5057 ± 50.50 pg/ml and 77200± 668.40 pg/ml with percent relevant errors of 3.4, 1.13 and 0.37%, respectively.

Ruggedness. The ruggedness of the method was assessed by comparison of the intra- and inter-day assay results for tamoxifen undertaken by two analysts. The %R.S.D. values for intra - and inter – day assays of tamoxifen in the cited formulations performed in the same laboratory by the two analysts did not exceed 4%, 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 (Heyden *et al.* 2001). As can be seen in table 2, the percent recoveries of tamoxifen were good under most conditions and did not show a significant change when the critical parameters were modified.

Recovery. A known amount of tamoxifen standard powder was added to the injection samples and then mixed, and was diluted to yield a starting concentration of 2000 pg/mL as previously described in sample preparation section. Afterwards, this solution was analyzed as previously described. The assay was repeated (n=9) for 3 consecutive days to obtain intermediate precision data. The observed concentration of tamoxifen was found to be 1993 ± 129 pg/ml. The resultant %R.S.D. was equivalent to 1.85% with a corresponding recovery percentage value of 99.90%.

Table 2. The influence of the changes in the experimental conditions on the performance of the FIA system.

Parameter	modification	Tamoxifen (% recovery)
pH	2.0	101.1
	2.3	99.9
	2.5	101.3
	3.0	100.0
flow rate ml/min	2.8	101.8
	3.0	101.1
	3.2	98.9
buffer composition (M)	0.04	98.9
	0.05	99.7
	0.06	101.5
Lab. Temperature (ºC)	20	101.6
	25	99.5
	30	100.3



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

Assay of tablets. The method developed in the present study was applied for the determination of tamoxifen in injections from the Iranian market. The results showed a percent recovery of 100.1% and a R.S.D. of 1.65%.

Determination of Tamoxifen in human urine and plasma.

The results of analysis of spiked human plasma (n = 5) and urine (n=5) is shown in table 3. The results are satisfactory, accurate and precise. No interference were noticed from the urine content after just dilution with the supporting electrolyte and as well as after plasma samples treatment. The major advantage of the method as applied to plasma and urine is that no prior extraction step is required.

Comparison of the sensitivity of the proposed method and other previously reported detection methods. Table 4. Compares the sensitivity (detection limit) of the proposed method with the other reported methods. As can be seen from table 4. the detection limit of this method is about 64 times lower than the best previously reported method .

Table 3. Application of the proposed method to the determination of Tamoxifen in spiked Human plasma and urine

Added (pg/mL)	Interpolated concentration	R.S.D (%)	R.E. (%)
2500 (plasma)	2400 ± 35	1.9	2.1
2000 (urine)	2100 ± 40	1.7	1.4

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

Table 4. The detection limit comparison of the methods

Method	Detection limit	Ref. No.
Capillary zone electrophores	3ng/ml	7
Capillary gas chromatographic	2.4 ng/ml	6
FFTCV	37pg/ml	This work

Acknowledgements

The authors express their appreciation to the Research Council of the University of Tehran for the financial support of this work.

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