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FFT-Adsorptive Voltammetric Technique for Pico-Level Determination of Tetracycline in Capsules at an Au Microelectrode in Flowing Solutions

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This study illustrates a novel method for fast monitoring of tetracycline in flow-injection systems. The fast Fourier transformation, along with continuous cyclic voltammetry (FFTCV) at a gold microelectrode in a flowing solution system, was used for the determination of tetracycline in its pharmaceutical formulation. The benefit of the developed technique is that it is 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.

Three considerable advantages are demonstrated by this method, which other methods do not possess. Firstly, there is no requirement for oxygen removal from the test solution; secondly, a picomolar detection limit is gained; and, finally, rapid determination of any such compound in a broad range of chromatographic methods.

As a result, the method was found to be linear over the concentration range of

16-440 pg/mL (r = 0.9962), with a limit of detection and quantitation of 4.5 and 16 pg/mL, respectively. Furthermore, the technique shows the requisite accuracy, sensitivity, precision, and selectivity to assay tetracycline in capsules.

Key Words: Tetracycline, ultramicroelectrode, continuous cyclic voltammetry, flow injection analysis, fast Fourier transformation.

Introduction

Tetracycline (TC) is an antibiotic that has been safely used to treat skin disorders for over 30 years (Figure 6). It is commonly used to treat acne and some other disorders,

such as rosacea and perioral dermatitis. Since TC does not cure, but only suppresses these skin disorders, it may be necessary to continue taking it for months or even years.[1]

TC is a broad-spectrum antibiotic used for medical purposes as well as in animal husbandry, either as a growth promoter in sub-therapeutic doses, prophylactically for disease prevention, or therapeutically

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for infection treatment.[2] Therefore, it is important to develop sensitive analytical methods for the measurement of degradation products in TC samples. A number of spectrophotometric and fluorimetric methods have already been used successfully for the determination of TC and its major degradation products.[3−5] Several liquid chromatographic methods have been described[6−9] and UV detection is used by the EP.[10] The sensitivity of chemiluminescence (CL) has spurred interest in the development of analytical methods for TCs.[11] Spectrophotometric methods are the most widely used for TC determination in bulk and pharmaceutical preparations.[12,13] Methods combining flow injection analysis with spectrophotometric detection have also been reported for tetracycline assessment.[14−17] There is, consequently, a continuing requirement for reliable, sensitive, and selective means for their determination. Procedures that have proved useful include electrochemical methods,[18,19] UV-visible spectrophotometry,[20−26] liquid chromatography,[27] and an immunoaffinity-based procedure.[28] Methods based on chemiluminescence generation have some advantages, such as high sensitivity and selectivity, as well as simple sample preparation and instrumentation.[29−30] Moreover, a tendency can be observed towards the development of miniaturized and fast methods to achieve highly-sensitive monitoring in the field of pharmaceutical, biomedical, and food analysis.

Recently, stripping voltammetric methods were used for the 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 requires fast analyte accumulation and fast potential sweeping, which is not appropriate for large electrodes.[32,33] As such, the introduction of UMEs has encouraged the use of voltammetric techniques. For example, they demonstrate steady state currents, have high sensitivity due to increased mass transport, and are applicable to highly resistant solutions. Additionally, UMEs have been used as sensors in various techniques, such as flow injection analysis,[34,35] cardiovascular monitoring, and organic compound analysis.[36−38] Nonetheless, this study aimed to present a novel method for the prompt determination of TC ultra trace amounts in its pharmaceutical preparation.

Experimental

Reagents

Reagents for preparation of the stock eluent solution for the flow-injection analysis were obtained from Merck Chemicals. Furthermore, TC capsules were purchased from a local pharmacy, each containing tetracycline hydrochloride equivalent to 250 mg of TC.

Moreover, all solutions were filled with the background electrolyte solution, used without the removal of the dissolved oxygen and prepared in double-distilled deionized water using analytical grade reagents.

Background electrolyte (BGE)

For preparation of the running buffer or BGE, 8.7 mL of phosphoric acid (85% w/v) was added to a 1000-mL volumetric flask. Afterwards, the resulting solution was diluted to a constant volume with distilled water. The pH was adjusted to 2.3 with sodium hydroxide. All solutions were freshly prepared each day and filtered using a Millipore filter (0.45 μm).

Standards and sample solutions

Standard stock solutions

Distilled water was used to make a standard stock TC solution (1 mg/mL), which was light-protected using foil. This solution was freshly prepared each day. It was stored at 4 °C for 1 day and was found to be stable during this period.

Standard solutions for Flow Injection Analysis (FIA)

An aliquot of the TC standard stock solution was diluted using distilled water several consecutive times. Afterwards, suitable aliquots of the latest diluted solution were dispensed into 10-mL volumetric flasks, which were filled with the running buffer to cover a final concentration range of 16-440 pg/mL.

Sample preparation

Preparation of the samples involved the following procedure. Twenty capsules were opened and their contents were weighed and mixed well. Then, powder, equivalent to the weight of one capsule, was accurately weighed into a 1-L volumetric flask, 500 mL of distilled water was added, shaken meticulously to dissolve, and filled and mixed totally. Suitable aliquots of this solution were filtered through a Millipore filter (0.45 μm). Then, 10 μL of the filtered solution was added to a 100-mL volumetric flask, which was filled with distilled water. In the end, 1 mL of the resulting solution was added to a 10-mL volumetric flask and filled with 0.05 M phosphoric acid to provide an initial concentration of 250 pg/mL.

Electrode preparation

Metal micro-wires (Goodfellow Metals Ltd., UK) were sealed into a soft glass capillary for the preparation of the gold UMEs (with a radius of 12.5 μm). Then, the capillary was cut perpendicular to its length to expose the wire. The electrode surface was polished for 1 min with extra fine carborundum paper and afterwards for 10 min with 0.3 μm alumina. Eventually, the electrode was washed with water before being placed in the cell.

A reference electrode of Ag (s) | AgCl (s) | KCl (aq, 1 M) was used for all measurements, while the auxiliary electrode was made of a Pt wire (1 cm long × 0.5 mm diameter). Silver epoxy (Johnson Matthey Ltd., UK) was used for the electrical contacts.

Flow injection setup

In Figure 1, the electrochemical cell of the flow -injection analysis is illustrated. For this analysis, a 10-roller peristaltic pump (UltrateckLabs Co., Iran) and a 4–way injection valve (Supelco Rheodyne Model 5020) with a 50-μL sample injection loop were used. Moreover, a plastic syringe was used for the introduction of the solutions into the sample loop. The flow rate of the eluent solution during the experiments was set to 3 mL/min and the cell volume was 100 μL.

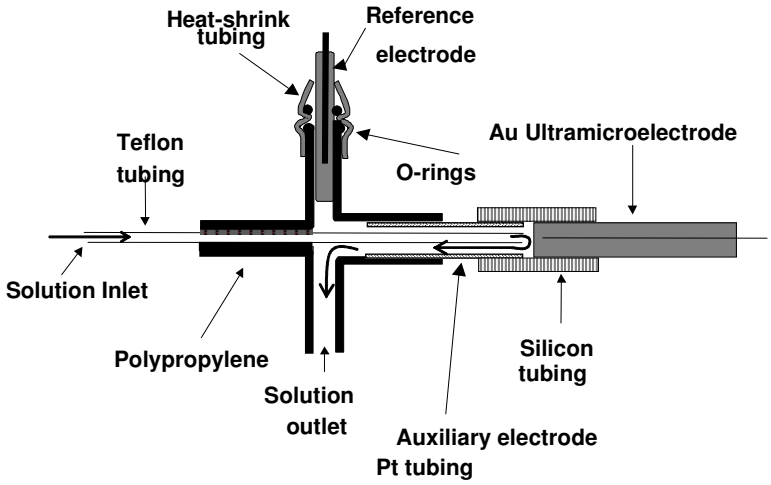

Figure 1. The electrochemical cell.

Data acquisition and processing

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

In Figure 2 the applied waveform potential diagram during the cyclic voltammetric measurements is shown. The potential waveform consists of 3 parts: a) potential steps, E_{c1} and E_{c2} (which are used for the oxidation and reduction of the electrode surface, respectively), during which the electrochemical cleaning of the electrode surface takes place; b) E_c, where the analyte accumulation takes place; c) the potential ramp, where the current measurements occur.

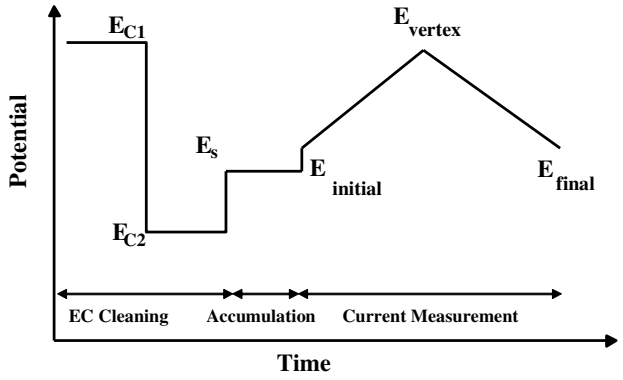

Figure 2. The applied potential waveform.

Here, the integration of the net current changes over the scanned potential range provides the signal calculation. In this case, the current changes (result of the injected analyte) at the voltammogram can be caused by various processes, which occur on the electrode surface. Those processes include oxidation and reduction of the adsorbed analyte, and 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 reduction peaks of the gold surface, the scan rate must be set high (e.g., > 20 V/s).

During the scan, some of the adsorbed analyte molecules are desorbed. Depending on the rate of those processes and the scan rate, the amount of the desorption analyte molecule (during the scan) can be changed. All the same, the adsorbed analyte molecules have not yet been removed from the electrode surface, leading to the redox inhibition of the electrode surface. Then, the ΔQ calculation is performed in agreement with all the current changes at the CVs.[39−43] Nonetheless, the selectivity and sensitivity of the analyte response, expressed in terms of ΔQ, strongly depends on the selection of the integration limits.

At this point of the study, the application of a special digital filtration should be mentioned. Initially, an electrode CV was recorded. Then, FFT was applied on the collected data and the existing high frequency noises were indicated. With the help of this information, the cutoff frequency of the analog filter was set at a certain value (where the noises were removed from the CV).

On the grounds that the crystal structure of a polycrystalline gold electrode greatly depends on the condition of the applied potential waveform,[42] various potential waveforms were examined in order to obtain a reproducible electrode surface (or a stable background signal). In fact, the application of cyclic voltammetry for the determination of electroactive compounds mainly faces low stability of the background signal due to the changes taking place in the surface crystal structure during the oxidation and reduction of the electrode in each potential cycle. After the examination of various potential waveforms, the best potential waveform, providing a stable background during the measurement, was the waveform depicted in Figure 2. As mentioned above, the potential waveform was continuously applied during an experimental run in which the collected data were filtered by the FFT method before use in the signal calculation.

The starting point for the electrochemical oxidation process of the gold surface is the hydroxyl ion electrosorption. In fact, at more positive potentials, it results in gold oxide formation, undergoing structural rearrangement.[44] Moreover, the surface oxidation initiation can occur with the water molecule adsorption, where AuOH is formed at a more positive potential, leading to a 2-dimensional phase formation of the gold oxide:

$$Au(H_2O) \longrightarrow AuO + 2e + 2H \tag{1}$$

Figure 3 (a and b) presents an example of the recorded CVs. Firstly, Figure 3a shows a CV sequence recorded during the flow analysis for the drug determination. The injection volume was of 50 μL of 1.0 $\times$ 10^{-6} M TC (in 0.05 M H_3PO_4) into the eluent solution containing 0.05 M H_3PO_4. On the graph, the time axis represents the time of the flow injection experiment. In the absence of TC, the shape of the CV curves is typical for a polycrystalline gold electrode in acidic media.[45] Secondly, Figure 3b illustrates the absolute current changes in the CV curves, after the subtraction of the 4 CVs' average background (in the absence of the analyte). In fact, it consists of a better way of presenting the electrode response since it provides more details about the adsorbed ion effect on the CV currents. Actually, the curves show that the current changes mainly take place at the potential regions of the oxidation and reduction of gold. When the electrode-solution interface is exposed to TC, which can be adsorbed on the electrode, the oxide formation process becomes severely inhibited. In detail, the surface process inhibition causes significant change in the currents at the potential region and, as a consequence, profound changes in the shape of the CVs take place. The universality of the detector is advantageous for chromatographic analysis, in which a mixture of compounds is present in the sample.

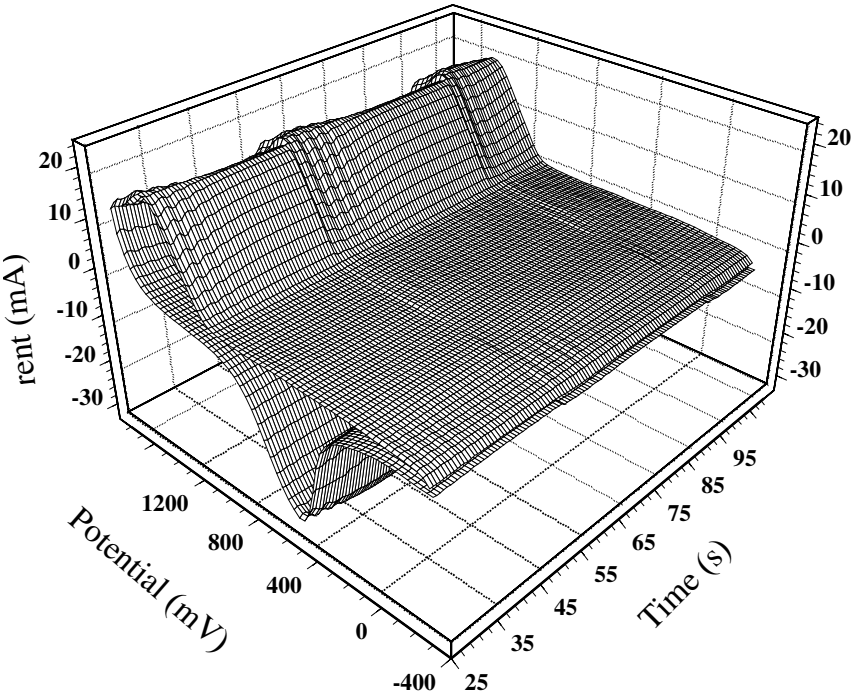

Figure 3a. Cyclic voltammogram at Au ultramicroelectrode recorded during a flow-injection experiment. The eluent was 0.05 M H_3PO_4, the flow rate was 100 $\mu\mu$L/s, and the sweep rate was 50 V/s. Each scan was preceded by a conditioning of 100 ms (at 1600 mV) and 100 ms (at 300 mV), respectively. The accumulation time was 600 ms at 500 mV. The injected solution (50 $\mu\mu$L) contained 1.0 $\times 10^{-6}$ M TC in 0.05 M H_3PO_4.

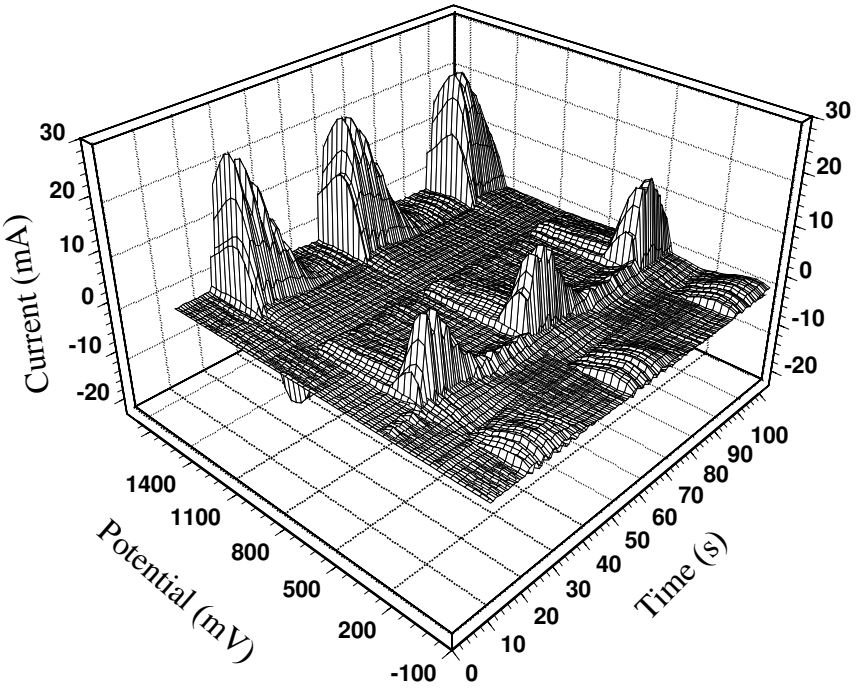

Figure 3b. Curves resulting from the subtraction of the CV average (in the absence of the analyte) from the CV test in (a).

Normally, the thermodynamic and kinetic parameters of adsorption, the mass transport rate, and the electrochemical behavior of the adsorbed species influence the analyte response. Regarding the free energy and the adsorption rate, they depend on the electrode potential, the electrode material, and to some extent on the choice of the concentration and the type of the supporting electrolyte. As a result, for the achievement of the detector maximum performance, the effect of the experimental parameters (such as the pH of the supporting electrolyte, the potential, the accumulation time, and the potential scan rate) must be examined and optimized.

Results and discussion

Optimization of the experimental parameters

The effect of eluent pH on the detector performance was additionally examined. The corresponding results are presented in Table 1. This Table explains that the best S/N ratio was obtained between pH 2 and 3. Moreover, it is illustrated that at pH > 9 the noise level in the baseline (ΔQ vs. time) is as much as 12% higher compared to that of the acidic solution.

Table1. The effect of pH on the microelectrode response.

pH	2.1	4	6	8	10	12
S/N	120	80	78	60	70	77

For the investigation of the influence of the scan rate and the eluent flow rate on the sensitivity of the detector response, solutions were injected having a TC concentration of 1.0×10^{-8} M. Afterwards, the detector responses to the injected sample were recorded at different scan rates (from 10 to 140 V/s) and eluent flow. These results are presented in Figure 4, exhibiting the maximum sensitivity at the scan rate of 50 V/s and flow rate of 3 mL/min.

It should be mentioned that the sweep rate effects influence the detection performance in 3 ways. The first influence concerns the speed in data acquisition. The second is the kinetic factors of the TC adsorption and the third is the eluent flow rate, which controls the time window of the solution zone in the detector.

In addition, the main reason for the application of high scan rates is desorption prevention of the adsorbed TC during the potential scanning (because under this condition the inhibition outcome of the adsorbed TC on the oxidation process can take place).

Indeed, the application of high scan rates is required for the use of this detection method in combination with fast separation techniques, such as capillary electrophoresis. Then, it is necessary to check how the method sensitivity is affected by the sweep rate. The employment of high sweep rates leads to the detection of the amount of adsorbed analyte on the electrode surface, since the potential scanning step is short in comparison with the accumulation period. Another significant factor is the time when the TC accumulation occurs at a potential that is greater or smaller than E_i. Nonetheless, the potential sweep rate is the determining factor that defines the sensitivity of the detection system, primarily because of the adsorption kinetic factors and the instrumental limitations.

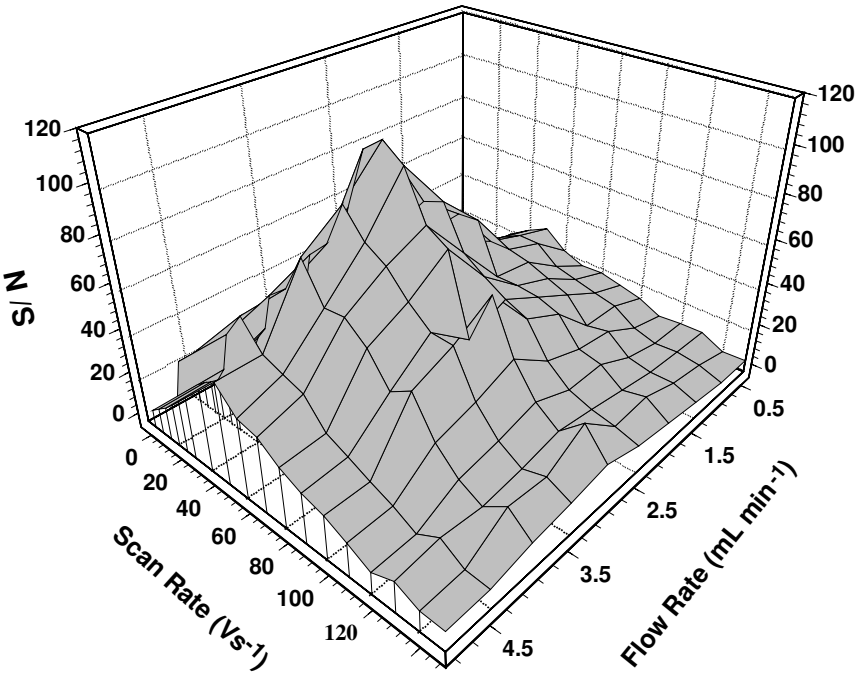

Figure 4. The effect of sweep rate on the response of the Au electrode (with a radius of 12.5 μm) to the injection of 1.0 X10^{-8} M TC in 0.05 M H_3PO_4 and the flow rate effect.

Apparently, the measurement sensitivity is greatly influenced by the changes in the parameters, which are related to the adsorption process, and they affect the applied potential, the time, and the potential of accumulation. For that reason, the influence of the accumulation potential and time on the response of the method for the injection of a solution of 1.0×10^{-8} M TC in 0.05 M H_3PO_4 was studied. In Figure 5, the detector response is depicted over the accumulation potential range of –600-1000 mV and the accumulation time range of 0.05-0.9 s. Clearly, the optimum conditions to be chosen are an accumulation potential of 500 mV and accumulation time of 600 ms, on the grounds that the electrode surface becomes TC-saturated within a 600 ms time window.

It has been already stated that the gold ultra microelectrode surface is small and in a short time the electrode surface can be saturated. Assuming that an appropriate potential is selected, the TC accumulation takes place on the electrode during the accumulation step. In fact, the existing differences in the kinetics of the electron transfer and mass transport result in diverse values for the saturation time of the various compounds.

Validation

The validation parameters of the method included selectivity, limit of quantitation (LOQ), limit of detection (LOD), robustness, accuracy precision, recovery, and linearity.[46–48]

Linearity

Linear regression analysis was applied along with the help of the least squares regression method for the evaluation of the linearity.[49,50] For the concentration range of 16-440 pg/mL, the calibration curves constructed for TC were linear. The peak areas of TC were plotted versus its concentration, and afterwards

linear regression analysis was carried out on the resultant curve. The results of this analysis provide a correlation coefficient of R = 0.9962 with % R.S.D. values ranging from 0.21% to 3.5% across the tested concentration range. Characteristically, the regression equation for the calibration curve was $Y = 4 \times 10^{10} X + 33.74$. Figure 6 presents the obtained calibration graph from the monitoring of TC in 0.05 M H_3PO_4.

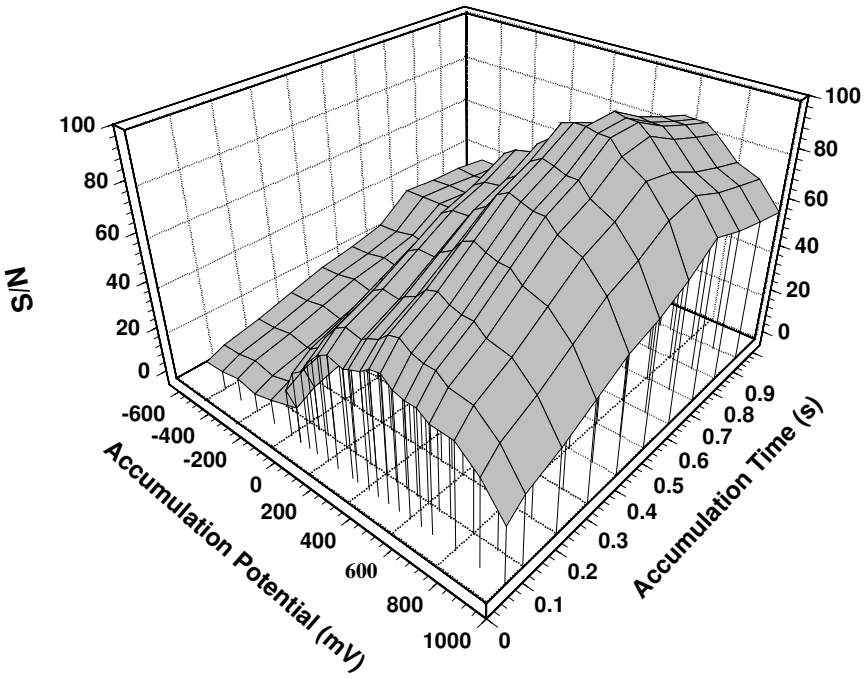

Figure 5. The potential effect and the accumulation time effect on the electrode response to the injection of 1.0×10^{-8}M TC in 0.05 M H_3PO_4.

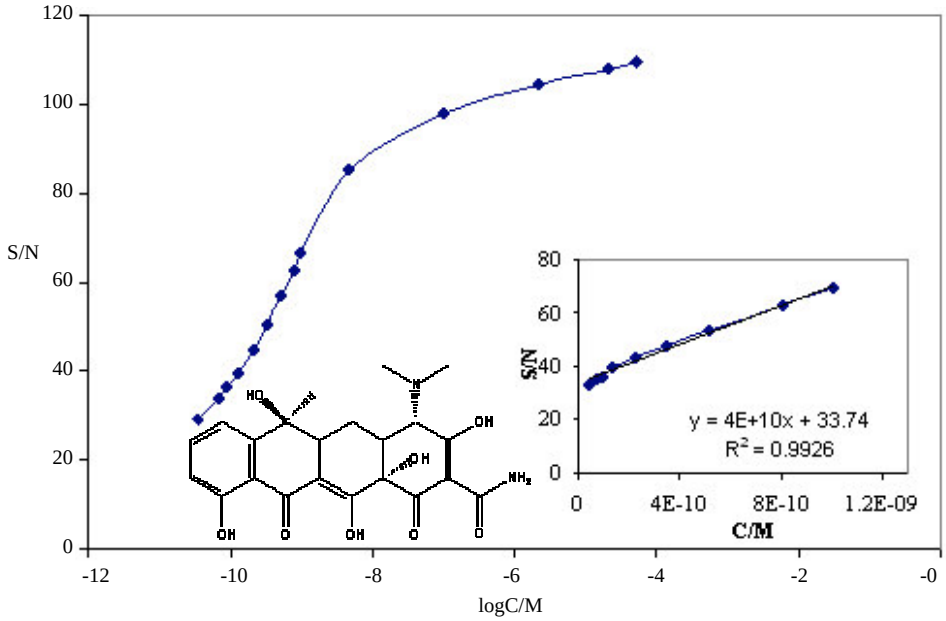

Figure 6. Calibration curves obtained for TC on the Au electrode in 0.05 M H_3PO.

LOQ and LOD

Firstly, the LOD was measured as the lowest analyte amount that may be detected to produce a response that is significantly different from that of a blank. The detection limit was approved by calculations, based on the standard deviation of the response (δ) and the slope (S) of the calibration curve at the levels approaching the limits, according to the equation LOD = 3.3 (δ/S).[51] The LOD for TC was equal to 4.5 pg/mL.

Secondly, the LOQ was measured as the lowest analyte amount that can be reproducibly quantified above the baseline noise, for which triplicate injections resulted in an RSD $\leq$ 2.25%. A practical LOQ, giving a good precision and acceptable accuracy, was equivalent to 16 pg/mL.

Precision

The precision assessment was performed in accordance with repeatability and reproducibility. Repeatability was investigated by injecting 9 replicate samples of each of the 16-, 80-, and 440-pg/mL standards in which the mean concentrations were 16.25, 79.6, and 443.6, with associated % R.S.D. values of 3.64, 1.24, and 0.24, respectively. Moreover, concerning the inter-day precision, it was assessed by injecting the same 3 concentrations for 3 consecutive days. In the end, the mean TC concentrations were 16.6, 83, and 445.5 pg/mL, and the associated % R.S.D. values were 3.14, 3.49, and 1.75, respectively.

Accuracy

For the accuracy assessment of the method, replicate (n = 6) peak areas of 3 accuracy standards (16, 80, and 440 pg/mL) were interpolated from a calibration curve, which was prepared as previously mentioned. Furthermore, the relevant error percentage and accuracy were calculated in each case. The resultant concentrations were 16.55 $\pm$ 0.6, 80.8 $\pm$ 1.07, and 442 $\pm$ 1.58 pg/mL, with relevant error percentages of 3.42%, 0.95%, and 0.43%, respectively.

Ruggedness

Afterwards, the ruggedness of the method was assessed. For this aim, a comparison was performed between the intra- and inter-day assay results for TC, which were made by 2 analysts. The % R.S.D. values for intra- and inter-day assays of TC in the cited formulations were performed in the same laboratory by the 2 analysts and they did not exceed 3.85%, therefore, demonstrating the ruggedness of the method.

The robustness was also examined while the parameters' values (the pH of eluent, the flow rate, the buffer composition, and the laboratory temperature) were slightly changed.[52] According to Table 1, the TC recovery percentages were satisfactory in most cases, without presenting any important changes during the alteration of the critical parameters.

Recovery

A known amount of tetracycline sulfate standard powder was added to the aliquots (n = 20) of the capsule contents. The resulting blend was mixed, extracted, diluted, and analyzed. The final nominal TC concentration was 269.5 pg/mL. The assay was repeated (n = 9) for 3 consecutive days to obtain the intermediate precision data. The resultant % R.S.D. value for this study was 1.3%, with a corresponding recovery percentage value of 99.81%.

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

Parameter	modification	Tetracycline (% recovery)
pH	2.0	101.1
	2.3	99.9
	2.5	101.4
	3.0	100.1
flow rate mL/min	2.8	101.7
	3.0	101.0
	3.2	99.94
buffer composition (M)	0.04	98.9
	0.05	99.8
	0.06	101.4
Lab. Temperature (°C)	20	101.5
	25	99.7
	30	100.2

Selectivity

The TC standard solutions were monitored in the presence of its formulation components in order to check the selectivity of the method. The responses were not different from those obtained in the calibration curve. As a consequence, it was concluded that the determination of TC in this formulation is considered to be free from component formulation.

Capsules assessment

The suggested technique was applied for the determination of TC in capsules from the Iranian market. The results illustrated a recovery percentage value of 100% and an R.S.D. value of 1.65%.

Sensitivity comparison of the detection methods

Eventually, the sensitivity (detection limit) of this method was compared with those of the previously reported methods in Table 3. In particular, the data in Table 3 revealed that the detection limit of this method is about 200 times lower than that of the most sensitive method.

Table 3. The detection limit comparison of the methods.

Method	Detection limit	Ref. no.
chemiluminescence	7200 pg/mL	20
spectrofluorimetric	1.2×10^4 pg/mL (inhuman serum albumin (HSA)	21
adsorptive stripping voltammetry	900 pg/mL	19
UV spectrophotometric flow-injection	69×10^6 pg/mL	23
flow injection chemiluminometric	5×10^5 pg/mL	29
U UV- Vis Spectroscopy	10,000 pg/mL	24
FFTCV	4.5 pg/mL	This work

Acknowledgments

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