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A Fast Stripping Continuous Cyclic Voltammetry Method for Determination of Ultra Trace Amounts of Nalidixic Acid

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In recent years, modern electroanalytical techniques, especially cyclic voltammetry, are becoming increasingly important in the determination of pharmaceutical products. This study presents a novel method for the determination of nalidixic acid (NA) in flow-injection systems, called fast stripping continuous cyclic voltammetry. This technique is simple, precise, accurate and time saving compared to similar methods. Initially, the effects of several method parameters on sensitivity were tested. Thus, the potential waveform was applied to an Au disk microelectrode in a continuous way. After a series of experiments, it was concluded that the best performance was achieved with the basic parameters set at pH = 2, sweep rate of 60 V s^{-1}, accumulation potential 100 mV and accumulation time 0.7 s. The detection limit was 0.07 pg mL^{-1} and the relative standard deviation was 2.2 % at the concentration of 10 nmol dm^{-3} for 10 runs. Major advantages of this method are (i) it is no longer necessary to remove oxygen from the test solution, (ii) fast determination of similar compounds by several chromatographic methods is possible, and (iii) the achieved detection limit is low.

Keywords
nalidixic acid
continuous cyclic voltammetry
fast Fourier transformation
flowing injection

INTRODUCTION

Nalidixic acid (NA) (1-ethyl-7-methyl-4-oxo-[1,8]naphthyridine-3-carboxylic acid, $C_{12}H_{12}N_2O_3$) is a narrow-spectrum synthetic antibacterial agent with bacteriostatic or bactericidal properties, depending on its concentration.[1,2] At its usual concentrations in clinical urine samples, this compound possesses antibacterial action against most gram-negative bacilli that cause infections of urinary ducts.

NA is more active against gram-negative than gram-positive organisms through the inhibition of their DNA gyrase.[3] The basic DC polarographic behavior of NA at DME was first studied by Starosak *et al*.[4]

The US Pharmacopoeia (USP)[5] recommends determining NA by titration with 0.1 M sodium methoxide in a non-aqueous medium (dimethylformamide), using thymolphthalein as indicator.

Measurements of low NA levels commonly rely on spectroscopy[6] or chromatography.[7,8] HPLC is the technique most frequently applied to NA detection either in dosage forms or in biological fluids.[9–12]

The fast stripping continuous cyclic voltammetry method is faster, easier, more sensitive and economical than

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spectrometric and LC methods. Moreover, the experimental methodology is less tedious and it can be successfully employed for material analysis in complicated systems (*e.g.,* tablets, urine, serum, *etc.*).

EXPERIMENTAL

Reagents

All solutions were prepared in double-distilled deionized water, filled with a background electrolyte solution and used without removal of dissolved oxygen.

Reagents for the preparation of running buffer or background electrolyte (BGE) solution for flow-injection analysis were acquired from Merck Chemicals. The nalidixic acid active substance was acquired from Eli Lilly Asia (Shanghai, China). NegGram® (Sanofi) tablets of 250 mg NA were bought from a local pharmacy.

Background Electrolyte

For the preparation of running buffer or BGE, 8.7 mL of phosphoric acid (w = 85 %) were added into a 1000 mL volumetric flask. After that, the resulting solution was diluted to a constant volume with distilled water. The pH was adjusted to 2 with sodium hydroxide. All solutions were every day freshly prepared and filtered using a Millipore filter (0.45 μm).

Standard Stock Solutions

A standard stock solution of NA (100 μg mL^{-1}) was prepared in the BGE. This solution was freshly prepared every day.

Standard Solutions for FIA

Aliquots of the NA standard stock solution were distributed into 10 mL volumetric flasks, which were filled up with running buffer to give a final concentration range of 0.23 to 23000 pg mL^{-1}.

Sample Preparation

Samples were prepared by weighing twenty tablets, which were then finely powdered and NA portions equivalent to 250 mg were transferred into 1000 mL volumetric flasks, shaken thoroughly to dissolve, filled up and mixed well. Suitable aliquots of this solution were filtered through a Millipore filter (0.45 μm) and 100 μL of the filtered solution was diluted with distilled water in a 500 mL volumetric flask. Finally, 1 mL of the resulting solution was added into a 10 mL volumetric flask and filled up with 0.05 M phosphoric acid to a concentration of 5000 pg mL^{-1}.

Preparation of Human Urine and Plasma Samples

Drug free human plasma was obtained from the Iranian blood transfusion service (Tehran, Iran) and stored at –20 °C until its use after gentle thawing. Urine was collected from healthy volunteers (males, around 35 years old).

For the determination of NA in human urine, 1 mL of untreated urine containing 5 ng mL^{-1} NA was placed into a 20 mL volumetric flask and diluted with a buffer solution, pH = 2, to the mark. Then, 50 μL of the aliquot were injected into the FIA system. For NA determination in plasma, 10 μL of aqueous NA solutions (5 ng mL^{-1}) was added to 1000 μL of untreated plasma. The mixture was vortexed for 30 s. To precipitate plasma proteins, plasma samples were treated with 20 μL of 20 % perchloric acid, $HClO_4$. After that, the mixture was vortexed for further 30 s and then centrifuged at 6000 rpm for 5 min. Eventually, a 50 μL aliquot of the obtained supernatant were injected into the FIA system. Voltamograms were recorded according to the above recommended procedure. Voltamograms of samples without NA did not show any signal that could interfere with direct determination and, therefore, external calibration could be used.

Electrode Preparation

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 (length 1 cm, diameter 0.5 mm). Silver epoxy (Johnson Matthey Ltd., UK) was used for electrical contacts.

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

Flow Injection Setup

The electrochemical cell of the flow-injection analysis is illustrated in Figure 1. For this analysis, a 10-roller peristaltic pump (UltrateckLabs Co., Iran) and a four-way injection valve (Supelco Rheodyne Model 5020) with a 50-mL sample injection loop were used. Further, a plastic syringe was used to introduce solutions into the sample loop. The flow rate of eluent solution during the experiments was set at 3 mL min^{-1} and the cell volume was 100 μL.

Data Acquisition and Processing

A potential waveform generally comprises three parts. First, the potential steps, E_{c1} and E_{c2}, when the electrode surface is oxidized and reduced, respectively. During these steps, the electrode surface is being cleaned electrochemically. Second, the E_{acc} step when the analyte is being accumulated. Third, there is the potential ramp step when current measurements are made. In Figure 2, the applied waveform potential diagram is represented throughout cyclic voltammetric measurements.

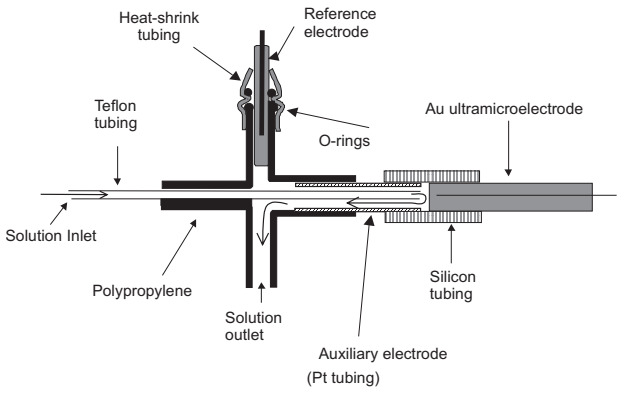

Figure 1. Schematic drawing of the electrochemical cell.

Many processes are observed on the electrode surface, causing current changes in the voltamogram (result of the injected analyte). These processes include: (i) oxidation and reduction of the adsorbed analyte, and (ii) oxidation and reduction inhibition of the electrode surface by the adsorbed analyte. Then, by integrating net current changes for the entire scanned potential range, the signal is calculated. Nonetheless, the scan rate must be set at high rates (*e.g.*, > 20 V s^{-1}) if the influence of the adsorbed analyte on oxidation and reduction peaks of the gold surface are desirable.

It should also be taken into account that some of the adsorbed analyte molecules are desorbed during the scan. As a consequence, the amount of the desorbed analyte molecules can be changed in proportion to the rate of the said processes and the scan rate. On the other hand, the adsorbed analyte molecules still remain on the electrode surface, leading to the red/ox inhibition of the electrode surface. Afterwards, the ΔQ calculation is done in agreement with all the current changes at the CVs.[17–20] The selection of integration limits defines the selectivity and sensitivity of the analyte response (ΔQ).

At this point, the application of special digital filtration should be mentioned. Initially, an electrode CV was recorded. Then, FFT was applied to the collected data and the existing high frequency noises were indicated. Based on this information, the cutoff frequency of the analog filter was set at a certain value (where the noises were removed from the CV). Several potential waveforms were investigated in order to obtain a reproducible electrode surface (or a stable background signal), because the crystal structure of a polycrystalline gold electrode greatly depends on the condition of the applied potential waveform.[21] Actually, the application of cyclic voltammetry to determination of electroactive compounds demonstrates low stability of the background signal, because of the changes taking place in the surface crystal structure during the electrode oxidation and reduction in each potential cycle. After examining many potential waveforms, the best potential waveform, providing a stable background during the measurement, was the one depicted in Figure 2. As already mentioned, the potential waveform was continuously applied during an experiment run and the collected data were filtered by the FFT method before their use in the signal calculation.

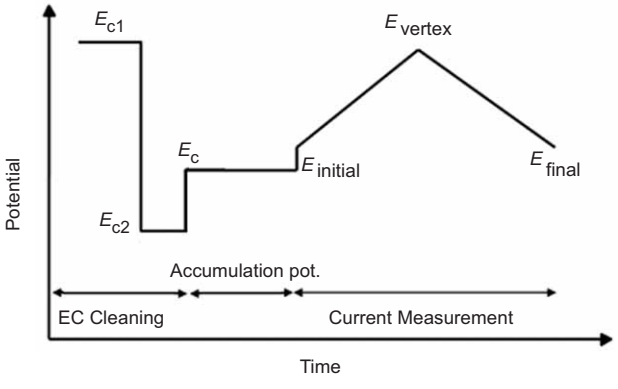

Figure 2. The potential waveform applied.

The starting point for the electrochemical oxidation process of gold surface is the hydroxyl ion electrosorption. In fact, at more positive potentials, it results in gold oxide formation, undergoing structural rearrangement.[22] Another way to initiate surface oxidation is the water molecule adsorption when AuOH is formed at a more positive potential, leading to a two-dimensional phase formation of gold oxide:

$$Au + H_2O \longrightarrow AuO + 2\ e^- + 2\ H^+ \qquad (1)$$

Figure 3 (a, b) gives an example of the recorded CVs. Figure 3a displays a CV sequence recorded during the flow analysis for drug determination. The injection volume was 50 μL of 1.0 μmol dm^{-3} NA (in 0.05 M H_3PO_4), which was injected into the eluent solution containing 0.05 mol dm^{-3} H_3PO_4. The time axis on the graph represents the time of the flow injection experiment. When NA is not present, the shape of CV curves is typical of a polycrystalline gold electrode in acidic media.[23] Figure 3b displays absolute current changes in CV curves, after subtraction of the average background of 4 CVs (in the absence of analyte). Involving a better way of presenting the electrode response, this provides more details about the adsorbed ion effect on CV currents. In reality, the curves show that current changes mainly take place in the potential regions of oxidation and reduction of gold. The oxide formation process becomes severely inhibited when the electrode-solution interface is exposed to NA, which can be adsorbed on the electrode. More precisely, the surface process inhibition causes significant changes to the currents in the potential region and, as a consequence, profound changes in the shape of CVs occur. In chromatographic analysis, where a mixture of compounds is present in the sample, the universality of the detector is beneficial.

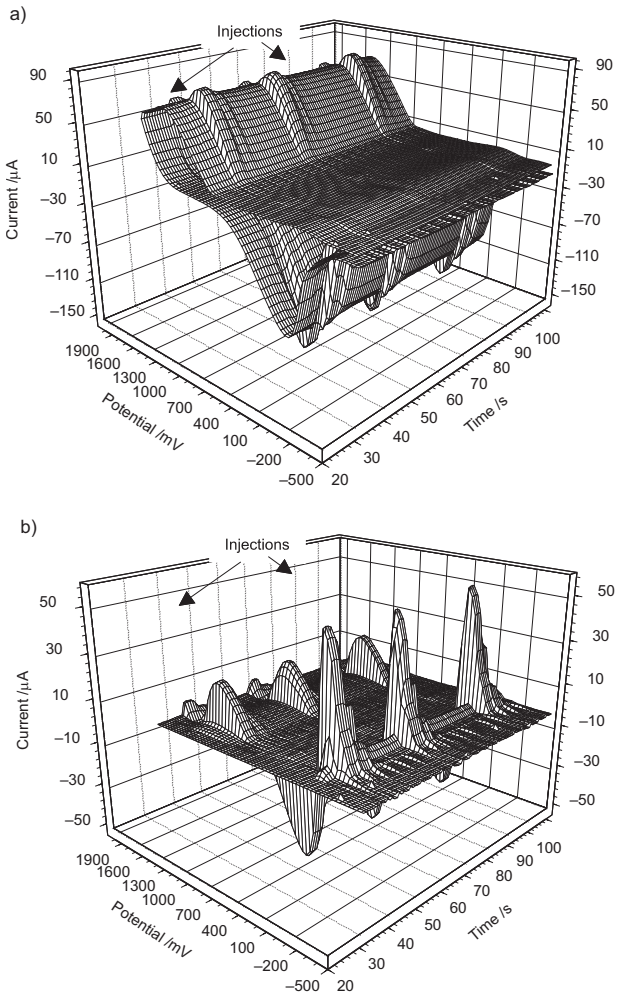

Figure 3. a) Cyclic voltamogram at a 12.5 μm Au ultramicroelectrode recorded during a flow-injection experiment. The eluent was 0.05 M H_3PO_4, the flow rate was 3 mL min^{-1}, and the sweep rate was 60 V s^{-1}. Each scan was preceded by 100-ms (at 1600 mV) and 100-ms (at 300 mV) conditioning, respectively. Accumulation time was 700 ms at 100 mV. Injected solution (50 μL) contained 1.0 μmol dm^{-3} nalidixic acid in 0.05 M H_3PO_4. The inset graph is the CV of background electrolyte (a) and after injection of nalidixic acid (b). b) Curves result from the subtraction of average CVs (in the absence of analyte) from the test of CVs in (a).

Thermodynamic and kinetic parameters of adsorption, the mass transport rate and the electrochemical behavior of the adsorbed species normally influence the analyte response. With regard to free energy and adsorption rate, they depend on the electrode potential, electrode material and, to some extent, on the choice of the concentration and the type of the supporting electrolyte.[24–30] As a result, to achieve maximum performance of the detector, the effect of experimental parameters (such as the supporting electrolyte pH, potential, accumulation time and the potential scan rate) must be examined and optimized.

For data acquisition, a setup of 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.

RESULTS AND DISCUSSION

Optimization of Experimental Parameters

First, the effect of eluent pH on the detector performance was monitored. The results are demonstrated in Figure 4. The figure shows that the highest ΔQ was obtained with pH = 2. Moreover, it is showed that, at pH values higher than 9, the ΔQ level in the baseline is higher by up to 12 % compared to that of acidic solution.

To investigate the influence of scan rates and eluent flow rate on the sensitivity of the detector response, solutions with a NA concentration of 9.0 nmol dm^{-3} were injected. Afterwards, detector responses to the injected sample were recorded at different scan rates (from 10 to 140 V s^{-1}) and eluent flow rates. The results are presented in Figure 5, exhibiting maximum sensitivity at the scan rate of 60 V s^{-1} and flow rate of 3 mL min^{-1}.

It should be noted that the sweep rate affects detection performance in a triple manner. The first influence concerns the speed of data acquisition. The second involves the kinetic factors of NA adsorption and the third is the eluent flow rate, which controls the time window of the solution zone in the detector.

Furthermore, the main reason for the application of high scan rates is to prevent desorption of adsorbed NA during potential scanning (because under such conditions, inhibition of the oxidation process by adsorbed NA may take place).

As a matter of fact, application of high scan rates is required for the use of this detection method in conjunction with fast separation techniques such as capillary electrophoresis. It is also necessary to check how the method sensitivity is affected by the sweep rate. Employment of high sweep rates allows detection of the ad-

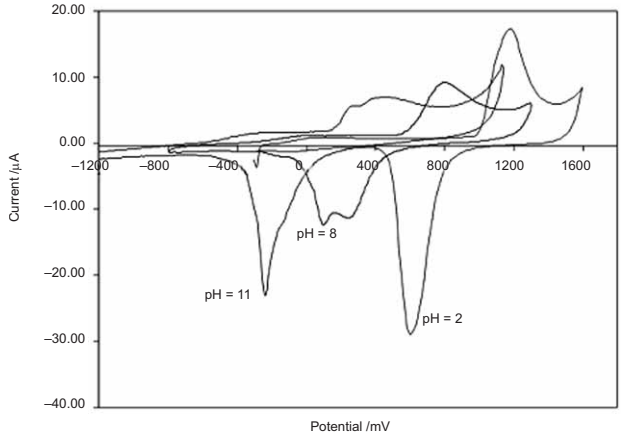

Figure 4. The effect of eluent pH on detector performance.

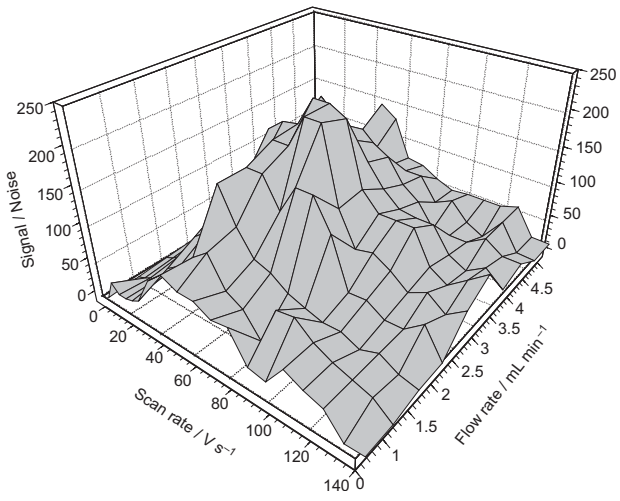

Figure 5. The effect of sweep rate on the response of Au electrode (radius 12.5 μm) to injections of 9.0 nmol dm^{-3} nalidixic acid in 0.05 M H_3PO_4 and the effect of flow rate.

sorbed analyte on the electrode surface because the potential scanning step is short compared to the accumulation period. Another significant factor is the time when the NA accumulation occurs at a potential that is greater or smaller than E_i. Owing mostly to the adsorption kinetic factors as well as the instrumental limitations, the potential sweep rate comprises the determining factor, defining the sensitivity of the detection system.

Obviously, the measurement sensitivity is strongly influenced by changes in the parameters related to the adsorption process; they affect the applied potential, the time and potential of accumulation. For this reason, the influence of the accumulation potential and time on the method response was studied for the injection of a NA solution of 9.0 nmol dm^{-3} in 0.05 M H_3PO_4. Figure 6 displays the detector response over the accumulation potential ranges (–400 to 800 mV) and the accumulation time range from 0.1 s to 1.0 s. It is easily observed that the conditions of choice are the accumulation potential of 100 mV and accumulation time of 700 ms.

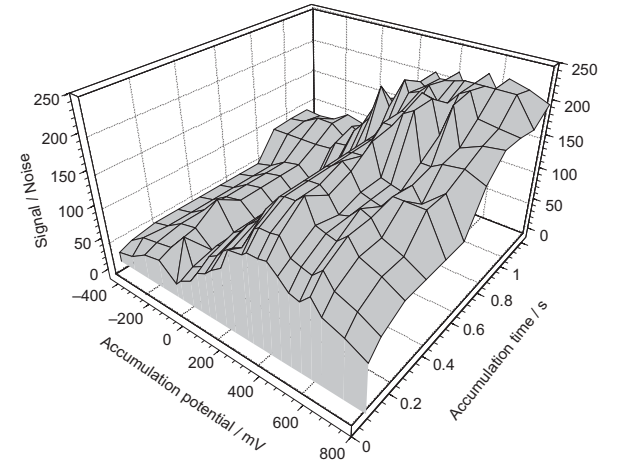

Figure 6. The effect of accumulation potential on the response of Au electrode to injections of 9.0 nmol dm^{-3} nalidixic acid in a 0.05 M H_3PO_4 and the effect of accumulation time.

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

Validation

The technique was validated with respect to linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, ruggedness/robustness, recovery and selectivity.[31–33]

Linearity

Linearity was evaluated by linear regression analysis, calculated using the least squares regression method.[34,35] Calibration curves constructed for NA were linear over the concentration range of 0.23–23000 pg mL^{-1}. Peak areas of NA were plotted *versus* its concentration and, after that, linear regression analysis was carried out of the resultant curve. The results of this analysis provide the correlation coefficient R = 0.977 with R.S.D. values ranging from 0.27–4.3 % over the analyzed concentration range. Figure 7 represents the calibration graph of NA determination in 0.05 M H_3PO_4. Typically, the regression equation for the calibration curve was found to be $y = 0.5808\ x + 60.94$.

LOQ and LOD

LOD was measured as the lowest detectable analyte amount to produce a response significantly different from that of a blank. The detection limit was confirmed by calculations, based on the standard deviation of the

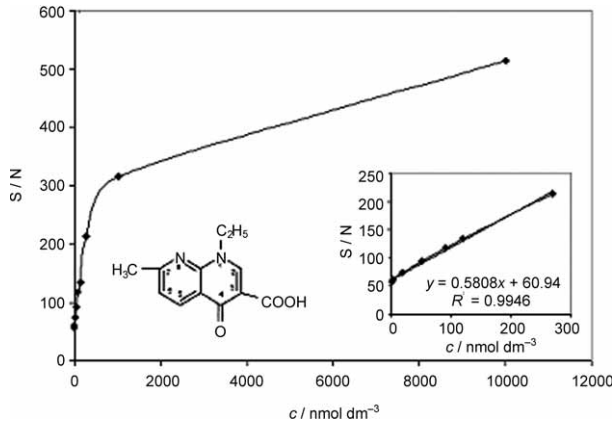

Figure 7. Calibration curves obtained for ranitidine on an Au electrode in 0.05 M H_3PO_4.

response (δ) and the slope (S) of the calibration curve at levels approaching the limits according to the equation LOD = 3.3 (δ/S).[36] The LOD score for NA was 0.07 pg mL^{-1}.

Secondly, LOQ was measured as the lowest analyte amount that can be reproducibly quantified above the baseline noise, for which triplicate injections resulted in R.S.D. ≤ 1.55 %. Practical LOQ, giving good precision and acceptable accuracy, was found to be equivalent to 0.23 pg mL^{-1}.

Precision

Parameters of repeatability and reproducibility were investigated in order to assess the precision of the technique. For repeatability monitoring, nine replicate samples of each of the 0.23, 5000 and 23000 pg mL^{-1} standards were injected. In the end, the mean concentrations were found to be 0.24, 5120 and 22900 with associated R.S.D. values of 3.9, 1.45 and 0.37 %, respectively. Regarding the inter-day precision, the same three concentrations were injected for 3 consecutive days, providing mean NA concentrations of 0.23, 5110 and 22920 pg mL^{-1} and associated R.S.D. values of 4.1, 2.5 and 1.12 %, respectively.

Accuracy

Replicate (n = 6) peak areas of three accuracy standards (0.23, 5000 and 23000 pg mL^{-1}) from a calibration curve were interpolated as already mentioned. In addition, the relevant error and accuracy were calculated in each case. The resultant concentrations were (0.24 ± 0.011) pg mL^{-1}, (5081 ± 96.5) pg mL^{-1} and (23103 ± 92.7) pg mL^{-1} with relevant error of 3.9, 1.64 and 0.45 %, respectively.

Ruggedness

Comparison of intra- and inter-day results was carried out for the estimation of ruggedness. These results, obtained by two analysts at the same laboratory, indicated that the corresponding R.S.D. values in the cited formulations did not exceed 4.5 %, thus demonstrating the ruggedness of the method.

On the other hand, robustness was examined while the parameters (eluent pH, flow rate, buffer composition and laboratory temperature) were being slightly changed.[37] Table I shows that the NA recovery were good under most conditions, not showing any significant change when critical parameters were modified.

Recovery

A known amount of NA standard powder was added to tablet samples and was then extracted, diluted and analyzed as previously described. In the end, the nominal NA concentration was found to be 5490 pg mL^{-1}. The assay was repeated (n = 9) for 3 consecutive days to obtain intermediate precision data. For this study, the resultant R.S.D. value was equivalent to 1.48 % with a corresponding NA recovery of 99.81 %.

TABLE I. Influence of changes in experimental conditions on the FIA system performance [a]

Parameter	Modification	NA recovery / %
pH	1.8	100.2
	2	101.4
	2.3	99.9
Flow rate / mL min^{-1}	2.8	100.1
	3.0	101.3
	3.2	99.9
Buffer conc. / mol dm^{-3}	0.04	99.9
	0.05	101.5
	0.06	100.8
Lab. temperature / °C	20	99.9
	25	101.0
	30	101.2

[a] NA, nalidixic acid.

Selectivity

This parameter was checked by monitoring the NA standard solutions in the presence of the formulation components. The responses were not different from those obtained in the calibration curve. Thus, it was proved that the determination of NA in this formulation can be considered to be free from component formulation.

Tablet Assessment

After the method was applied to Iranian commercial tablets, the resulting data showed a NA recovery of 99.90 % and the respective R.S.D. value of 1.89 %.

Determination of Nalidixic Acid in Real Samples

The proposed method was also applied to the determination of NA in spiked urine and plasma samples. Results of the analysis of spiked human plasma (n = 5) and urine

TABLE II. Application of the proposed method to determination of NA in spiked human plasma and urine[a]

Added NA pg mL^{-1}	Interpolated concentration	R.S.D. %	R.E. %	Standard method [b]
50 (plasma)	49.90 ± 0.03	3.15	3.50	50.15 ± 0.02
400 (urine)	400.02 ± 0.10	2.20	3.20	400.50 ± 0.02

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

[b] USP, Ref. 5.

TABLE III. Comparison of the detection limits of some detection methods

Method	LOD / pg mL^{-1}	Reference
Micellar electrokinetic capillary chromatography	200	13. – Separation and simultaneous determination of nalidixic acid, hydroxynalidixic acid and carboxynalidixic acid in serum and urine by micellar electrokinetic capillary chromatography
Cathodic adsorptive stripping voltammetry	766	14. – Cathodic adsorptive stripping voltammetric determination of nalidixic acid in pharmaceuticals, human urine and serum
Reversed-phase HPLC	35×10^6	15. – Simultaneous separation and determination of five quinolone antibiotics using isocratic reversed-phase HPLC: Application to stability studies on an ofloxacin tablet formulation
Spectrofluorometry	1214	16. - Spectrofluorometric determination of certain quinolone antibacterials using metal chelation
FFTCV	0.07	This work

(n = 5) are shown in Table II. The results were satisfactory, accurate and precise. No interference was recorded from the urine content, directly after dilution with the supporting electrolyte or after the treatment of plasma samples. 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 Detection Methods

Finally, Table III lists and compares the detection limits (sensitivity) of detection techniques. As it is immediately evident, the detection limit of this method is superior to all the previously reported ones, about 2850 times lower than that of the most sensitive method.

CONCLUSIONS

This work demonstrates that the concentration of NA in flowing solution can be measured indirectly *via* monitoring the current changes in oxidation and reduction of the electrode surface. In this method, fast Fourier transform continuous cyclic voltammetry (FFTCV), the S/N ratio is enhanced by using a fast Fourier transform of the analyte and signal integration. Also, to improve the sensitivity, the method takes advantage of analyte adsorption on the Au microelectrode and the influence of adsorbed impurity, if any, in the eluent was removed by background subtraction. FFTCV can be considered to be a new sensitive, accurate and fast method for determination of similar drugs, with the ability of adsorption to gold surface, in chromatographic systems such as HPLC and CE. Finally, such a detection limit (at nanomolar level) makes the method suitable for bio-analysis. For instance, the method was applied to determination of NA in tablet form and biological samples and showed good agreement with the reported values. However, experimental parameters should be optimized in order to obtain better sensitivity for a specific drug. This method can be used for ultra trace determination of other surface active substances after optimization of effective parameters.

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SAŽETAK

Metoda brze kontinuirane cikličke voltametrije za određivanje tragova nalidiksične kiseline

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Moderne elektroanalitičke tehnike, a posebno ciklička voltametrija, postale su u posljednje vrijeme veoma važne za analizu farmaceutskih proizvoda. Ovaj rad opisuje novu metodu određivanja nalidiksične kiseline korištenjem protočnog sustava. Metoda se naziva brza kontinuirana ciklička voltametrija. U usporedbi sa sličnim metodama, ova je jednostavna, precizna, točna i brza. U prvom dijelu rada istraženi su utjecaji nekoliko parametara metode na osjetljivost. Potencijal zlatne disk mikroelektrode kontinuirano se mijenja. Najbolji rezultati postižu se kod pH = 2, uz brzinu promjene potencijala 60 V s^{-1}, potencijala akumulacije 100 mV i vremena akumulacije 0,7 sekundi. Granica detekcije je 0,07 pg mL^{-1}, a relativna devijacija je 2,2 % ako je koncentracija 10 nmol dm^{-3} uz deset ciklusa. Prednosti metode su da nije potrebno uklanjati kisik iz otopine, da se može primijeniti na slične spojeve u kombinaciji sa kromatografskim tehnikama i da je granica detekcije niska.