Application of a Continuous Square-Wave Potential Program for Sub Nano Molar Determination of Ketotifen

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An electroanalytical method has been developed for the determination of the ketotifen by continuous square wave adsorptive stripping voltammetry on a ultra-gold microelectrode (Au UME) in aqueous solution with phosphate buffer as supporting electrolyte. The best adsorption conditions were found to be pH 2.3, an accumulation potential of 300 mV (Au *vs.* Ag: AgCl–KCl 3 M) and an accumulation time of 400 ms. Variation of admittance in the detection process is created by inhibition of oxidation reaction of the electrode surface, by adsorption of ketotifen. Furthermore, signal-to-noise ratio was 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 square-wave frequency, eluent pH, and accumulation time were optimized. Effects of square-wave frequency, step potential and pulse amplitude were examined for the optimization of instrumental conditions. The calibration curve is linear in the range 2.0×10^{-7}—5.0×10^{-12} M with a detection limit of 2.0×10^{-12} M (*ca.* 0.7 pg/ml). The method maybe applied direct determination of the drug in pharmaceutical and biological samples. For a concentration of 5.0×10^{-8} M a recovery value of 99.89% is obtained.

Key words fast Fourier transformation; square-wave voltammetry; gold ultramicroelectrode; flow-injection; ketotifen

Ketotifen fumarate [KTF; 10*H*-benzo(4,5)cyclohepta(1,2-*b*) thiophen-10-one, 4,9-dihydro-4-(1-methyl-4-piperidinylidene)-(*E*)-2-butenedioate] is a nonspecific, oral mast cell stabilizer. Its main biochemical pharmacological activities are H receptor antagonism, phosphodiesterase inhibition and inhibition of calcium flux in smooth muscle preparations.[1] It is applied to prevent the development of allergic conjunctivitis[2,3] asthma[4,5] and even showed an anti-wrinkle effect.[6] Asthma is a substantial health problem among children and adults worldwide, with high and increasing prevalence rates in many countries. More than 7% of Iranian children (4 million) are suffering from asthma according to the first National Asthma Prevalence Study. Oral salbutamol, aminophylline and ketotifen have been commonly used for asthma management. Ketotifen is one of the rare drugs that has been extensively used and shown safe in children. Ketotifen is an antihistamine. Histamine can produce allergy symptoms such as sneezing, runny nose, and watery eyes. Ketotifen is used to treat itching of the eyes caused by allergy to dust, pollen, animals and other allergens.

For determination of ketotifen several methods such as ultraviolet–visible (UV–vis) spectrophotometry,[7,8] high performance liquid chromatography (HPLC),[9] gas chromatography-mass spectrometry (GC-MS)[10] and liquid chromatography-mass spectrometry (LC-MS)[11] has been used.

The combination of microelectrode (ME) with square-wave voltammetry (SWV) has recently been shown advantageous for environmental detection of several compounds.[12] The adaptation of this technology to adsorptive stripping voltammetry (ASV) of ketotifen on a gold ME could provide a substantial improvement for rapid analysis.[13,14] This paper describes a fundamentally different approach to SWV measurement, in which the detection limits are improved, while preserving the information content of the SW voltammogram. The approach is designed to separate the voltammetric signal and background signal in frequency domain by using discrete fast Fourier transformation (FFT) method. Further improvement in the signal was gained by two-dimensional integration of the electrode response over a selected potential range and time window of the signal. Although at sufficiently high scan rates cyclic voltammetry (CV) can approximate an ac voltammetric technique and can be used to investigate electrode surface phenomena such as physical adsorption, FFT-SWV may be a more appropriate technique for monitoring analyte adsorption, since the potential dependence of analyte adsorption may be more clearly characterized. SWV measures the current response while rapid alternating potentials are applied during a staircase scan whereas cyclic voltammetry which uses a forward and reverse linear dc scan is not sensitive to the potential dependence of changes that occur in the double layer.

Experimental

Reagents All solutions were prepared in double-distilled water using analytical-grade reagents. Reagents in use in preparation of the stock eluent solution for flow-injection analysis (0.05 M Phosphoric acid and NaOH 1 M used for adjusting pH of the eluent) were obtained from Merck Chemicals. In all experiments all solutions were made up in the background electrolyte solution, and were used without removal of dissolved oxygen.

Assay Sample Preparation Twenty tablets were weighed and finely powdered and portions equivalent to 1 mg ketotifen were transferred into 100 ml volumetric flask; 50 ml distilled water was added, shaken thoroughly to dissolve, made up to volume and mixed well. Suitable aliquots of solution were filtered through a Millipore filter (0.45 μm). One microliter of the resulting solution was added to a 10 ml volumetric flask and made up to volume with 0.05 M phosphoric acid to yield starting concentration of 3.0×10^{-8} M.

Determination of Ketotifen in Human Urine and Plasma Two milliliter untreated urine containing 20 ng/ml ketotifen was placed into a 10 ml volumetric flask and diluted with water to the mark. One milliliter of this aliquot solution was diluted with pH 2 buffer solution to 5 ml into a volumetric flask. Then a 50 μl aliquot was injected into the flow injection analysis (FIA).

For the determination of ketotifen in plasma, 100 μl aqueous ketotifen solutions (0.8 ng/ml) were added to 100 μl of untreated plasma. The mixture was vortexed for 30 s. To precipitate the plasma proteins, the plasma samples were treated with 20 μl perchloric acid $HClO_4$ 15%. After that, the mix-

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ture was vortexed for a further 30 s then centrifuged at 6000 rpm for 5 min. Then, 50 μl aliquot of the obtained supernatant was injected into the FIA system. The voltammograms were recorded according to the above recommended procedure. The voltammograms of samples without ketotifen did not show any signal that could interfere with the direct determination, so external calibration could be used.

Apparatus 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 (with a length of 1 cm and diameter of 0.5 mm).

All electrochemical experiments were done using a setup comparing a personal computer (Pentium IV) equipped with a data acquisition board (PCL-818H, Advantech Co.) that was used to output an analog waveform to the working electrode and acquire current readings from the working electrode that connected to a custom-made potentiostat. The algorithms used to interpret the current response from each waveform cycle were discussed previously.[12] Most of the waveform parameters could be modified from within the software; including the pre- and post-scan potential/time, square-wave frequency/amplitude, dc ramp initial/final potential, and ramp time.

The equipment for flow injection analysis (FIA) included a 10-roller peristaltic pump (Home made) and a four-ways injection valve (Supelco Rheodyne Model 5020) with a 50 μl sample loop. Solutions were introduced into the sample loop by means of a plastic syringe. The electrochemical cell used in flow-injection analysis was the same as shown in our last papers. The flow rate of eluent solution in all experiments described in this paper was 0.5 ml/min.

Results and Discussion

A technique that was found to improve detection performance was adding pre- and post-scan pulses to the applied SWV wave form. In this new method to improve the detector sensitivity, the FFT-SWV technique was modified in the potential excitation waveform and current sampling and data processing. The potential waveform consisted of three sections: a) electrode conditioning; b) accumulation part and c) measurement of the potential waveform contained three additional potential steps, E_{c1} to E_{c2} (for cleaning the electrode surface) and E_{acc} (for accumulation of ketotifen). As shown in Fig. 1, the measurement part of the waveform contains multiple SW pulses with amplitude E_{sw} and frequency of f_o, being superimposed on a staircase potential function, which was changed by a small potential step of ΔE (the difference between every step of potential with the next one). The values of potential pulse of SW (E_{SW}) and ΔE were in the range of a few mV (10 to 50 mV). In potential ramp, the currents sampled four times per each SW polarization cycle.

Figure 2 shows the changes in the electrode admittance, of the gold electrode in 0.05 M H_3PO_4 into the eluent solution, caused by the injection of a solution of 100 μl of 1.0×10^{-6} M ketotifen. The FFT-SW modulation had an amplitude of 40 mV and a frequency of 750 Hz. Before each scan, the electrode was held at E_{c1} potential (1400 mV) for 60 ms, the E_{c2} potential at -200 mV for 60 ms then accumulation potential; E_{acc} at 300 mV for 400 ms.

The single peak at potential 1000 mV at the voltammogram is due to redox of gold to make change at current as equation below:

$$2Au+3H_2O\rightarrow Au_2O_3+6H^++6e^-$$

When the electrode potential passes the zero charge potential, changes in the double layer capacitance are caused by the reorientation of the water molecule and ion exchange at the Helmholtz layer.[13] Since such processes (*e.g.* water molecule reorientation) are very fast, the pseudo capacitance peak can be observed easily in various electrolytes even at frequencies >1 MHz. The second peak, with a shoulder, is related to oxidation of the electrode.

In Fig. 3a the peak current change is clear during the time after injection of analyte. The analyte signal appears as a current decline in certain potential at the FFT-SWVs admittance. This result from inhibition of the electrode surface processes by the adsorbed ketotifen. The differential form of the voltammograms is shown in Fig. 3b. In the differential graphs, it can be also noted that the analyte signal extends over a potential range of the FFT-SWV.

Adsorption phenomenon is much more general than red/ox reactions. Adsorption of organic molecules onto a gold surface can be an extremely complex process, which also involves the desorption of adsorbed water molecule, hydroxyl, or electrolyte anions. Although there will be some dependence on the structure of the molecule, adsorption onto metal electrodes will occur in varying degrees with most organic compounds. Moreover, adsorption of species onto an electrode has been avoided in flowing systems since it causes electrode fouling and analyte carryover. Such changes in the electrode surface concentrations and diffusion layer conductivity will result in a change in the charging current response. When FFT-SWV is used to monitor a flowing system, analyte adsorption will cause a measurable change in the admittance response. In addition to this, faradic FFT-SWV current may result from chemisorptions of the analyte onto the gold surface.

Norouzi and coworkers[14—30] reported a detection method by using flowing systems CV to monitor the adsorption of several inorganic and organic molecules onto Au electrode.

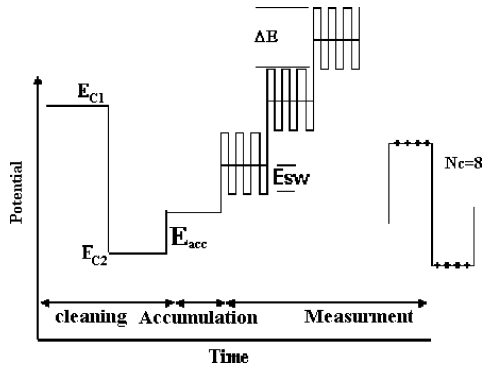

Fig. 1. Schematic of Potential Wave Form in Continuous Square-Wave Voltammetry

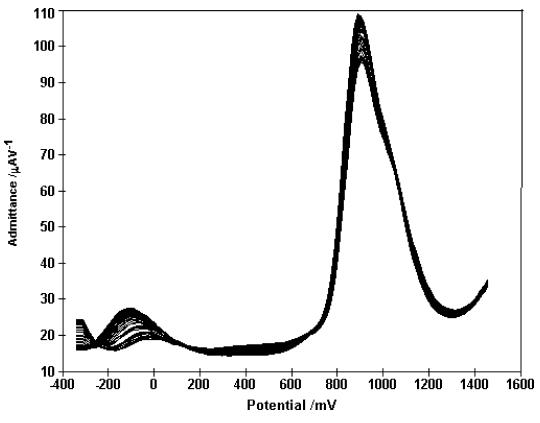

Fig. 2. Square Wave Voltammogram of 1.0×10^{-7} M Ketotifen in H_3PO_4 0.05 M at an Au UME at the Frequency 750 Hz

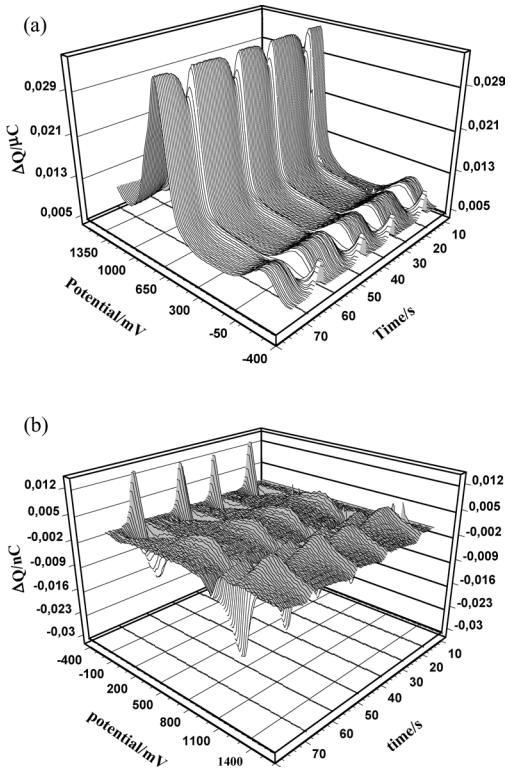

Fig. 3. (a) FFT Square-Wave Voltammograms at a 25 μm Au UME Recorded during a Flow-Injection Experiment

The eluent was 0.05 M H_3PO_4; the flow rate was 0.5 ml/min, and the frequency was 600 Hz. The injected solution (50 μl) contained 1.0×10^{-6} M ketotifen in 0.05 M H_3PO_4. (b) Average of Five Au FFT SW Voltammograms (in 0.05 M H_3PO_4) Subtracted from the Displayed Voltammograms

Similarly, using FFT-SWV technique, these current responses (in response to an alternating potential) can be examined to determine the time-dependence of the change in the current response due to analyte adsorption. It should be noted that in this method all processes studied involve adsorption of analytes; hence both charging and faradic currents may potentially carry useful analytical information. It is advantageous in FFT-SWV to collect more current samples near the end of the forward and reverse pulses and use signal averaging to increase the S/N (Signal/Noise). It is well known that, in the traditional Osteryoung SWV method[31—33] the current is sampled at two points for each square wave, t_1 (the end of the first SW pulse) and t_2 (the end of the second SW pulse). The difference current [(current at t_2)−(current at t_1)] for each square wave is plotted *vs.* dc ramp potential to obtain a peak-shaped voltammogram for an electroactive species. In the Osteryoung technique, the majority of the charging current will have decayed at the end of each pulse, allowing the faradic current to be sampled independently. FFT-SWV is able to sample the current across the entire SW period and use a selected portion of the forward and reverse voltammogram to calculate the difference current to increase faradic current such as traditional SWV. One of the advantages of scanning approaches for this electrochemical detection is that different parts of voltammogram can be selected for calculating the detector signal, based on response integration. A total absolute difference function (ΔQ) can be calculated using the following equation:

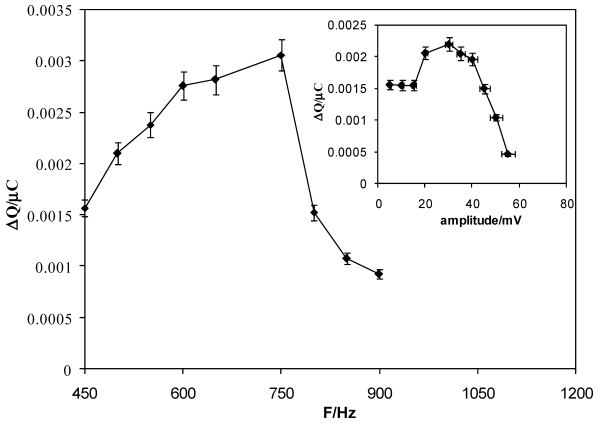

Fig. 4. Effect of Frequency and Amplitude on the Signal-to-Noise Ratio of Au UME Response to the Injection of 3.0×10^{-8} M Ketotifen in 0.05 M H_3PO_4

$$\Delta Q(s\tau) = \Delta t\left[\sum_{E=E_i}^{E=E_v}|A(s,E)\cdot E - A(s_r,E)\cdot E| + \sum_{E=E_v}^{E=E_i}|A(s,E)\cdot E - A(s_r,E)\cdot E|\right] \quad (1)$$

Where, s is the sweep number, τ is the time period between subsequent sweeps, Δt is the time difference between two subsequent points on the FFT-SW curves, $A(s,E)$ represents the admittance of the FFT-SW curve recorded during the s-th sweep and $A(s_r,E)$ is the reference admittance of the FFT-SW curve. E_i and E_v are the initial and the vertex potential, respectively. The reference FFT-SW curve was obtained by averaging a few FFT-SW curves (10 to 30) recorded at the beginning of the experiment (*i.e.* before injection of the analyte). By optimizing accumulation potential and time, the square-wave amplitude and frequency and selecting suitable flow rate of the FFT-SW response, the ΔQ adsorption-based response can be maximized.

Optimization of FFT-SW Frequency and Amplitude To study the effect of the above factors, the SW frequency range 500—900 Hz and amplitude 5 to 50 mV were examined at a concentration of 3.0×10^{-8} M of ketotifen. In Fig. 4 the importance of frequency and amplitude is demonstrated for solutions of ketotifen. In fast voltammetric analysis, the SW frequency and amplitude are important factors since analyte signal, background noise, and peak shape rely on speed of excitation signal. It should be noted that the solution resistance, electrode diameter, and stray capacitance of the system will limit the sensitivity gains obtained by raising the SW frequency. However, increasing the SW frequency will increase the SW peak current, or the sensitivity, but this will be tempered by a higher charging/faradic current ratio. Due to this absorption, the SW frequency acts similar to sweep rate in CV. For example, in this method to achieve a scan rate $\geq$10 V/s the SW frequency should be $>$500 Hz (assuming an increment of 20 mV with each SW cycle). Therefore using very high SW frequencies causes a shorter potential scan times; consequently, the response peak for the flow analysis becomes smaller and skewed due to insufficient time for oxidation of the electrode surface. While application of lower SW frequencies results in a longer potential scan, which results in a lower number of potential scan for each injected

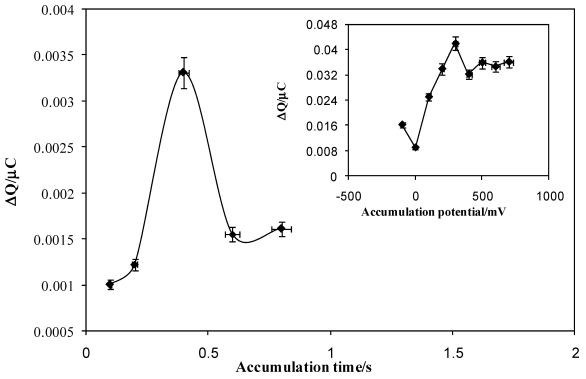

Fig. 5. Effect of Accumulation Time and Potential on the Response of 25 μm Au UME to the Injection of 3.0×10^{-8} M Ketotifen in 0.05 M H_3PO_4

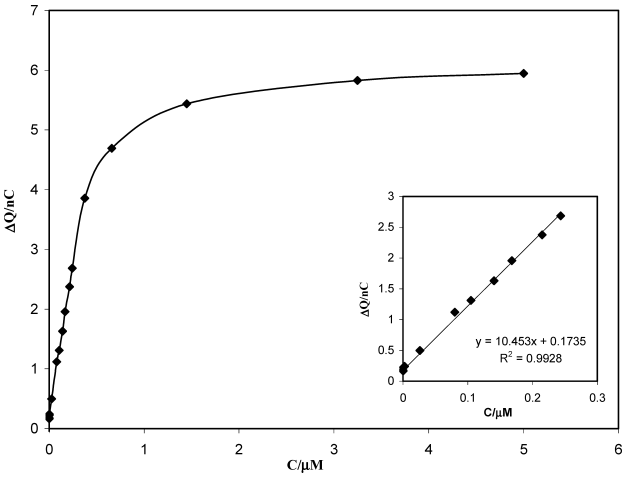

Fig. 6. Calibration Curve for Ketotifen in 0.05 M H_3PO_4 under Optimum Conditions at Flow-Injection System

sample zone. A series of SW frequencies was examined to determine the optimal frequency for the detection of ketotifen. A plot of SW frequency *vs.* S/N showed that a frequency of 800 Hz was the instrumental limit for this system. Above this frequency, excessive charging currents interfered with measurement of the faradic current, decreasing the ketotifen ΔQ. Thus further studies of SWV detection used 1000 Hz with a dc ramp time of 100 ms to provide an overall sample rate of 20 Hz.

Theoretically, the optimal square-wave amplitude for a reversible system is 50/n mV in which n is the number of electron.[34] To determine the influence of SW amplitude on ΔQ, various amplitudes were investigated. Figure 4 shows the effect of SW amplitude on increasing ΔQ. It was observed with increasing amplitude until 30 mV ΔQ was increased and after that ΔQ began to decrease when amplitudes greater than 30 mV were used. Low frequency noise (baseline drifting) was more pronounced when SW amplitudes above 10 mV were used. SW amplitude of 30 mV and frequency of 750 Hz were found optimal.

Effect of Accumulation Potential and Time In Fig. 5 the importance of accumulation time and potential is demonstrated for solutions of ketotifen. Similar to stripping voltammetric techniques, in this method the high sensitivity is mainly attributed to analyte pre-concentration *via* physical, chemical, or electrochemical adsorption. In this method, it is also possible to make use of the observation that many analytes will adsorb at electrodes at suitably applied potentials, E_s. To optimize the performance of FFT-SWV detection in FIA, the effect of accumulation time on electrode response and peak shape was evaluated. An accumulation time range of 0.1—0.9 s and an accumulation potential range −500 to 900 mV were examined.

If analytes cannot be accumulated in the selected time, then some loss in ΔQ would be expected. Therefore to reach a maximum surface coverage, or maximum sensitivity, the accumulation time should be long enough. However, long accumulation times also reduce the number of data points (at least 10 data points are need to plot analyte peak) for plotting the electropherogram; thus accumulation times of 400 ms were selected as a compromise between maximum ΔQ and minimum peak broadening. The losing data point was noted when accumulation time was >400 ms, likely due to short retention time of analyte zone in the electrochemical cell for the flow analysis. Therefore after data analyzing the accumulation time 400 ms and accumulation potential 300 mV were chosen.

Calibration Curves Figure 6 illustrates the obtained calibration curves for measurements of ketotifen in 0.05 M H_3PO_4. The experimental conditions were set at optimum values so as to obtain the best detection limits. The results shown in Fig. 6 represent the integrated signal for 3 to 5 consecutive flow injections of the standard solution.

As mentioned above, the electrode response could be expressed in various ways as peak heights or peak areas. For this reason, the magnitude of the flow-injection peaks depends on the choice of the data processing methods. Like stripping voltammetry methods, here, the electrode response is proportional to the electrode coverage. This assumption, however, may be less obvious when inhibition of oxide formation by adsorbents (ketotifen is considered, but not unlikely). For example, it has been shown that a decrease of charge in the oxide formation region on gold caused by adsorption of species is proportional to the surface coverage.[35] Measurements carried out for small analyte concentrations allow estimation of the detection limit C_{DL}:

$$C_{DL} = \frac{3s_b}{\text{slope}}$$

Where s_b is the standard deviation (or noise) of the baseline around the flow-injection peak.

The linearity was evaluated by linear regression analysis, calculated by the least square regression method.[36] The calibration curves constructed for ketotifen were linear over the concentration range of 5.0×10^{-11}—2.5×10^{-7} M. Peak areas of ketotifen were plotted *vs.* concentration and linear regression analysis were performed on the resultant curve. A correlation coefficient of $R=0.9968$ with %RSD. It was found values ranging from 0.25—3.6% across the concentration range studied were obtained following linear regression analysis. Typically, the regression equation for the calibration curve was $Y=10.453X+0.1735$. Figure 6 shows the calibration graph that obtained for the monitoring of ketotifen in 0.05 M H_3PO_4. The limit of detection (LOD) was measured as the lowest amount of analyte that may be detected to produce a response that is significantly different from that of a blank. Limit of detection was approved by calculations based on the

Table 1. Application of the Proposed Method to the Determination of Ketotifen in Spiked Humane Plasma and Urine

Added (pg/ml)	Interpolated concentration	RSD (%)	RE (%)
400 (plasma)	395.2±22.5	2.24	1.2
800 (urine)	805.35±20.0	2.11	0.67

Data obtained from five replicates at each concentration. Interpolated concentration data expressed as mean±S.D. RE is relative standard error. The relative error is the absolute error divided by the magnitude of the exact value. The percent error is a version of the relative error.

Table 2. Comparison of the Detection Limit of the Proposed Method with the Other Reported

Method	Detection limit	Ref.
LC-MS	20.0 ng/ml	11
GC-MS	0.01 ng/ml	10
Colorimetric	0.32 μg/ml	8
Chemiluminescence	3.0 ng/ml	12
FFT-SWV	*ca.* 0.7 pg/ml	This work

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 (δ/S).[36] The LOD for ketotifen was 2.0×10^{-12} M. The limit of quantitaion (LOQ) was measured as the lowest amount of analyte that can be reproducibly quantified above the baseline noise, for which triplicated injections resulted in an RSD≤1.75%. A practical LOQ giving good precision and acceptable accuracy was found to be 4.0×10^{-12} M.

Analytical Application After application of the method to the Iranian market injection, the resulting data showed a recovery percentage value of 99.88% and a respective RSD value of 1.92%. The proposed method was also applied to the determination of ketotifen in spiked urine and plasma samples. The results of analysis of spiked human plasma (n=5) and urine (n=5) are shown in Table 1. The results are satisfactory, accurate, and precise. No interference was noted from the urine content after just dilution with the supporting electrolyte. 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 Method and Other Previously Reported Methods Table 2 compares the detection limit of the proposed method with other reported methods. As is immediately obvious, the sensitivity of the method is superior to all previously reported methods. The data in Table 2 shown that the detection limit of the method is about *ca.* 20 times lower than the most sensitive reported method.

Conclusion

This report describes a novel, sensitive, and widely applicable FFT-SWV FIA detection method using of UME gold electrode. FFT-SWV was demonstrated to provide sensitive detection of a wide range of analytes based on oxidation of the electrode surface. Currently, work is progressing on the enhancement of the detection electronics and FIA cell to allow incorporation of a second sensing electrode positioned away from the detection zone which will enable automatic, analog subtraction of the background response. It is hoped that this will make FFT-SWV easier to use as well as provide enhanced sensitivity. Also, application of FFT-SWV to high-performance liquid chromatography is being considered.

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