Scientific paper

Non-electroactive Recognition: Pico-level Monitoring of Flunixin by Its Sub-second Adsorption at Au Microelectrode by Fast Fourier Transforms Continuous Cyclic Voltammetric Technique (FFTCCV)

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Abstract

In this work a novel method for the determination of flunixin 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 flunixin 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 30V s^{-1}, accumulation potential of –100 mV and accumulation time of 0.4 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 14–266400 pg/ml (r = 0.9975) with a limit of detection and quantitation 4 and 14 pg/ml, respectively. **We used this method to determination of flunixin in tablets.**

Keywords: Flunixin, ultr-amicroelectrode, fast fourier transform cyclic voltammetry, flow injection.

1. Introduction

Flunixin, Figure 6, was used for the treatment of ulmonarydiseases and endotoxin-induced mastitis, and an MRL value of 40 μg kg^{-1} in milk for its metabolite5-hydroxyflunixin was set in Council Regulation2377/90.

Non-steroid anti-inflammatory agents (NSAID) are used in the treatment of bovine coliform mastitis in order to reduce the severity of the clinical signs, to improve cure and to minimise adverse effects of the disease.[1,2] The NSAID registered for veterinary use in Belgium are pyrazolone derivatives (e.g. phenylbutazone), nicotinic acid derivatives (e.g. flunixine meglumine), fenamates (e.g. tolfenamic acid), arylpropionic acid derivatives (e.g. carprofen, ketoprofen) and the oxicam group (e.g. meloxicam).[3] As we were interested in residues of NSAID in milk, we selected two compounds, flunixin meglumine and ketoprofen, for this study, because these compounds are licensed for milk cows and a withdrawal time of 12 h after the last treatment was indicated in the instructions. Flunixin is used for the treatment of pulmonary diseases and endotoxin-induced mastitis, and an MRL value of 40 μg kg^{-1} in milk for its metabolite hydroxyflunixin was set in Council Regulation 2377/90.

A number of analytical methods have been developed for plasma and other biological fluids dogs and horses.[4–10] A few methods have been applied to study the pharmacokinetics of flunixin in lactating cattle after single and multiple intramuscular and intravenous administration,[11,12] and to detect flunixin in milk[13] and in muscle tissue.[14]

An accurate, reliable, and reproducible assay was developed and validated to determine flunixin in bovine liver, kidney, muscle, and fat. The overall recovery and percent coefficient of variation (% CV) of twenty-eight determinations in each tissue for flunixin free acid were 85.9% (5.9% CV) for liver, 94.6% (9.9% CV) for kidney, 87.4% (4.7% CV) for muscle, and 87.6% (4.4% CV) for fat. The theoretical limit of detection was 0.1 μg/kg (ppb, ng/g) for liver and kidney, and 0.2 ppb for muscle and fat. The theoretical limit of quantitation was 0.3, 0.2, 0.6, and 0.4 ppb for liver, kidney, muscle, and fat, respectively.[15] A liquid chromatographic atmospheric pressure ionization ion spray method is described for the determination of flunixin (FLU), tiamulin hydrogen fumarate (TIA) in meat.[16]

In the pharmaceutical, biomedical and food analysis, a tendency toward the development of miniaturised and fast methods to achieve the high-sensitive monitoring, can be observed. Recently, stripping voltammetric methods were used in determination of heavy metal ions and some organic compounds, in the flowing solutions with a sensitivity range of parts-per-billion. Indeed, application of techniques like this requires fast accumulation of the analyte and fast potential sweeping which is not appropriate for large electrodes.[17,18] So the use of voltammetric techniques has been further stimulated by the advent of UMEs, e.g. their steady state currents, higher sensitivity due to the increased mass transport, and their ability to be used in solutions with very high resistance. UMEs have, for instance, been applied as sensors in various techniques such as flow injection analysis,[19,20] cardiovascular monitoring and analysis of organic compounds.[21–23] In this work, we wish to introduce a novel method for fast determination of ultra trace amounts of flunixin in its pharmaceutical preparation.

2. Experimental

2. 1. 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. Flunixin standard powder was purchased from Spidaj Mexican Company. Flunixin injections containing a label claim of 50 mg/ml flunixin in 50 ml & 100 ml vials that was purchased from a local pharmacy.

2. 2. 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 (0.45 μm) each day.

2. 3. Standards and Sample Solutions

2. 3. 1. Standard stock solutions

A standard stock solution of flunixin (1mg/ml) was prepared in methanol – water (50–50% v/v). This solution was freshly prepared each day.

2. 3. 2. Standard Solutions for FIA

Aliquots of standard stock solution of flunixin were dispensed into 10 ml volumetric flasks and the flasks made up to volume with the running buffer to give final concentrations range of 14–266400 pg/ml.

2. 3. 3. Assay Sample Preparation

A 1 ml aliquot of the injectable solution equivalent to 50 mg parent compound was added into a 1000 volumetric flask, made up to volume with distilled water and mixed well. 10 μl of the filtered solution was added to a 100 ml volumetric flask and made up to volume with 0.05 M phosphoric acid to yield starting concentration of 5000 pg/ml.

2. 4. 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, 1 cm in length and 0.5 mm in diameter.

2. 5. 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 flow?injection 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 100 μl/s.

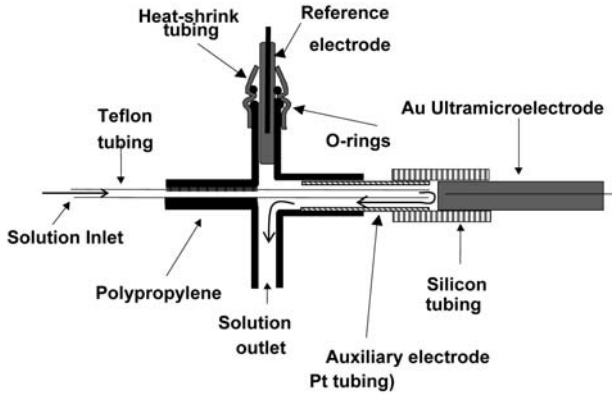

Figure 1. Diagram of the electrochemical cell

2. 6. 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.

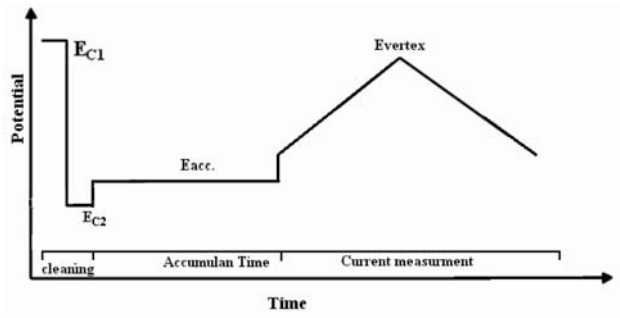

Figure 2. Diagram of the applied potential waveform

Signal Calculation in this method is established based on the integration of net current changes over the scanned potential range. It must be noted that in this case, the current changes (result of injected analyte) at the voltammograms can be caused by various processes, which take place at the electrode surface. Those processes include; a) oxidation and reduction of adsorbed analyte, and b) inhibition of oxidation and reduction of the electrode surface by the adsorbed analyte. Indeed, in order to see the influence of the adsorbed analyte on the oxidation and reductions peaks of the gold surface, the scan rate must be set at very high rates (e.g. >20 V/s)

However, during the scan, some of the adsorbed analyte molecules are desorbed. Depending on the rate of those processes and scan rate, the amount of the desorption analyte molecule (during the scan) can be changed. 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.[24–29] 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,[30] 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.[31] The surface oxidation can be initiated by adsorption of water molecule and then at more positive potential AuOH forms leading to the formation of a two-dimensional phase of gold oxide;

$$Au\ (H2O) \rightarrow AuO + 2e + 2H+ \quad (1)$$

An example of recorded CVs is shown in Figure 3 (a, b). Figure 3a. shows a sequence of CVs recorded during the flow analysis for determination of the drug. The volume of the injection was of 50 μL of 5.0×10^{-6} M flunixin (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 flunixin, 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 flunixin, which can adsorbed on the electrode, the oxide formation process becomes strongly inhibited. In fact, the inhibition of the surface process causes significant change in the currents at the potential region, and as a consequence the profound changes in the shape of CVs take place. Universality of the detector in this mode is very advantageous for chromatographic analysis, where a mixture of compounds presents in sample.

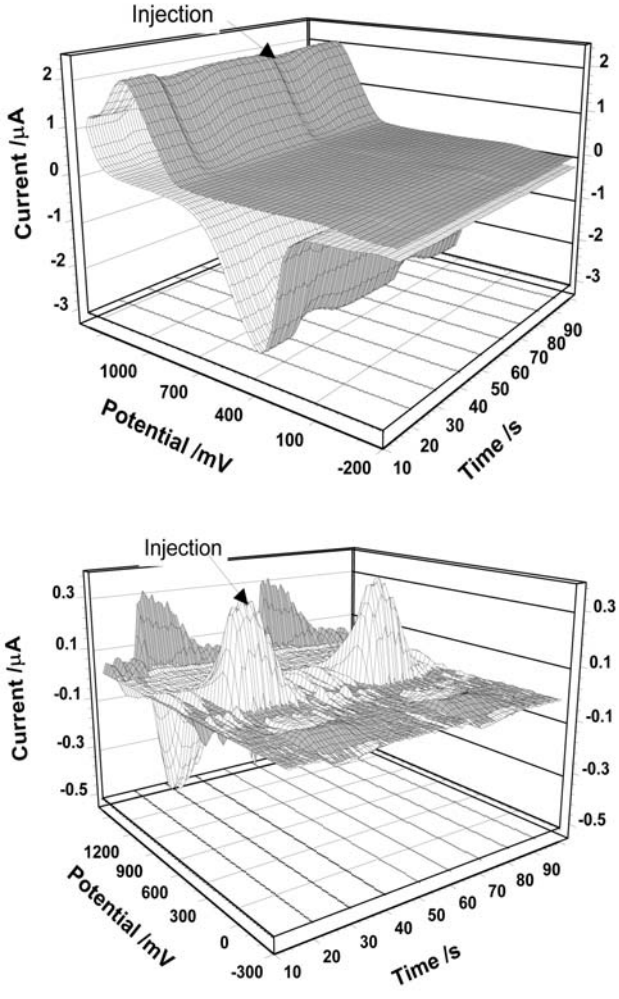

Figure 3. (a) Cyclic voltammograms at Au ultra-microelectrode recorded during the flow injection of 50 μL of 5.0×10^{-6} M of flonixin at optimum conditions. The eluent was 0.05 M H_3PO_4 and the flow rate was 3 ml/min. (b) Curves result from subtracting an average CV (in the absence of analyte) from test of the CVs in (a).

It must be noted that, theoretically, in this method, the analyte response can be affected by the thermodynamic and kinetic parameters of adsorption, the rate of mass transport and electrochemical behavior of the adsorbed species. The free energy and the rate of 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 accumulation and potential scan rate) must be examined and optimized.[32–47]

3. Results and Discussion

3. 1. 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

Also, in order to investigate the influence of scan rates and the eluent flow rate on the sensitivity of the detector response, solutions having a concentration of 5.0×10^{-7} M flunixin were injected. At different scan rates (from 5 to 80 V/s) and the eluent flow, the responses of the detector to the injected sample were recorded. The results are presented in Figure 4. As it is clear from the Figure 4, the detector exhibits the maximum sensitivity at 30 V/s of scan rate and 3 mL/min of the flow rate. The effects of the sweep rate on the detection performance can be taken into consideration from three different aspects: first, speed in data acquisition, second, kinetic factors of adsorption of the flunixin, 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 flunixin during the potential scanning, (because under this condition, the inhibition outcome of the adsorbed flunixin 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 ra-

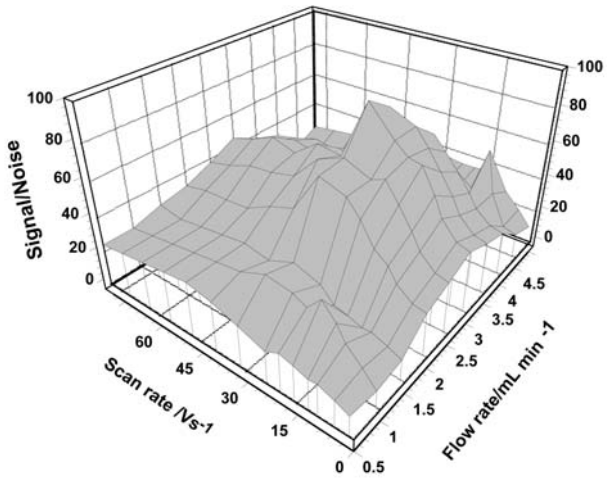

Figure 4. Effect of the sweep rate on the response of the Au microelectrode to injections of 5.0×10^{-7} M flonixin in 0.05 M H_3PO_4 and the effect of flow rate

tes. 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 flunixin 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 flunixin, in 0.05 M H_3PO_4, was studied. Figure 5. shows the detector response over the accumulation potential ranges 400 to –700 mV and accumulation time range from 0.05 s to 0.9 s. Based the figure accumulation potential –100 mV at time 400 ms was chosen as the optimum condition. Because, the surface of the electrode becomes saturated with the flunixin within 400 ms time window.

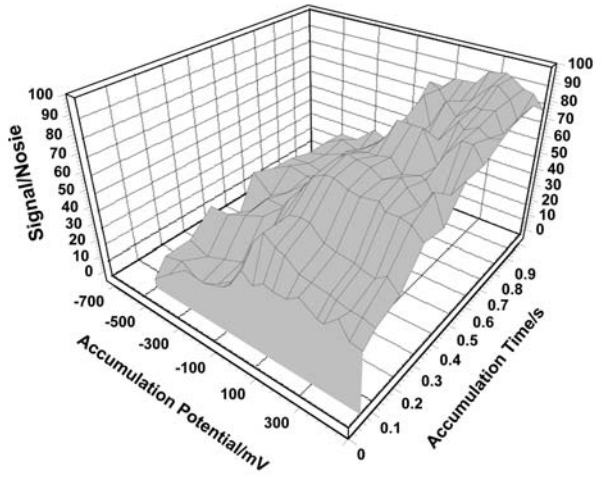

Figure 5. The effect of accumulation potential and the effect of accumulation time on the electrode response to injections of 5.0×10^{-7} M flonixin in 0.05 M H_3PO_4.

On the electrode, the accumulation of flunixin 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 flunixin in concentration between 14–1.2 10^5 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.

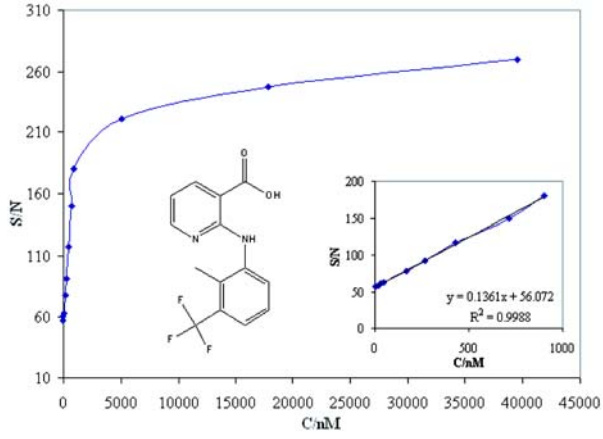

Figure 6. Calibration curves obtained for flonixin on the Au microelectrode in 0.05 M H_3PO_4.

3. 2. 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.[48–50]

3. 3. Linearity

The Linearity was evaluated by linear regression analysis, which calculated by the least square regression method.[21,52] The calibration curves constructed for flunixin were linear over the concentration range of 14.80–

266400 pg/ml. Peak areas of flunixin 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 0.25–3.90% across the concentration range studied were obtained following linear regression analysis. Typically, the regression equation for the calibration curve was found to be Y = 0.1361X + 56.072. Figure 6. shows the calibration graph that obtained for the monitoring of flunixin in a 0.05 M H_3PO_4.

3. 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.[53] The LOQ was found to be 14 pg/ml with a resultant % R.S.D. of 0.26% (n = 5). The LOD was found to be 4 pg/ml.

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

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

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

3. 5. 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 14, 5000 and 266400 pg/ml standards where the mean concentrations were found to be 14.35, 5095 and 267400 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 flunixin of 14.53, 5080 and 267680 pg/ml and associated % R.S.D. of 3.25, 3.70 and 2.1%, respectively.

3. 6. Accuracy

Accuracy of the assay was determined by interpolation of replicate (n = 6) peak areas of three accuracy standards (14, 5000 and 266400 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 14.48 ± 0.56 pg/ml, 5057 ± 50.50 pg/ml and 267385 ± 668.45 pg/ml with percent relevant errors of 3.4, 1.13 and 0.37%, respectively.

3. 7. Ruggedness

The ruggedness of the method was assessed by comparison of the intra- and inter-day assay results for flunixin undertaken by two analysts. The % R.S.D. values for intra – and inter – day assays of flunixin 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.[54] As can be seen in table 2, the percent recoveries of flunixin were good under most conditions and did not show a significant change when the critical parameters were modified.

3. 8. Recovery

A known amount of flunixin standard powder was added to the injection samples and then mixed, and was diluted to yield a starting concentration of 7000 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 flunixin was found to be 6993 ± 129 pg/ml. The resultant % R.S.D. was equivalent to 1.85% with a corresponding recovery percentage value of 99.90%.

3. 9. Selectivity

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

3. 10. Assay of Tablets

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

3. 11. Determination of Flonixin in Human Urine and Plasma

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

For the determination of flonixin in human urine, 10 ml of untreated urine containing 50 pg/ml flonixin was placed into a 100 ml volumetric flask and diluted with pH 2 buffer solution to the mark. Then 50 µl aliquot was injected into the FIA system.

For the determination of flonixin in plasma, 100 µl aqueous flonixin solution (10 pg/ml) was added to 100 µl of untreated plasma. The mixture was vortexed for 30 s. In order to precipitate the plasma proteins, the plasma samples were treated with 20 µl perchloric acid $HClO_4$ 20%. After that, the mixture was vortexed for a further 30 s and then centrifuged at 6000 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 flonixin do not show any signal that can interfere with the direct determination, so external calibration can be used.

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.

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

Added (pg/mL)	Interpolated concentration	R.S.D (%)	R.E. (%)
10 (plasma)	10.1 ± 0.3	2.6	2.0
5 (urine)	4.9 ± 0.4	1.5	1.3

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

3. 12. 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 25 times lower than the best previously reported method.

Table 4. The detection limit comparison of the methods

Method	Detection limit	Ref. No.
liquid chromatography in bovine muscle tissue	6000pg/ml	14
high–pressure liquid chromatography in plasma and milk	50000pg/ml	11
HPLC/TM in liver	100 pg/ml	15
LC /MS	5000pg/g	16
FFTCV	4pg/ml	This work

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Povzetek

Razvili smo novo metodo za določanje flunixina v pretočni analizi z adsorpcijo na Au mikroelektrodi s kontinuirno ciklično voltametrijo s hitro Fourierovo transformacijo. Metoda je preprosta za uporabo, točna, natančna ter časovno nezahtevna. Raziskali smo učinke različnih parametrov na občutljivost metode. Najboljše rezultate smo dobili pri pH 2, hitrosti preleta 30 V s^{-1}, potencialu akumulacije –100 mV in času 0,4 s. Prednosti pred obstoječo metodo so predvsem v tem, da ni potrebno odstranjevanje kisika izsistema ter pikomolarna meja zaznavnosti. Linearnost smo določili v območju 14-266400 pg/ml (r = 0,9975) z mejo zaznavnosti 4 pg/ml in mejo določljivosti 14 pg/ml. Metodo smo uporabili za določevanje flunixina v farmacevtskih izdelkih.