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Determination of the antibacterial trovafloxacin by differential-pulse adsorptive stripping voltammetry

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Abstract

A differential-pulse adsorptive stripping voltammetric method for the determination of trace amounts of the antibacterial trovafloxacin (TRFLX) is proposed. The optimal experimental parameters for the drug assay were: accumulation potential = −0.30 V (vs. Ag/AgCl), accumulation time = 120 s, pulse amplitude = 50 mV and scan rate = 5 mV s^{-1} in Britton–Robinson buffer (pH 4.5). The linear concentration range of application was 2.0–20.0 ng ml^{-1} of TRFLX, with a relative standard deviation of 3.6% (for a level of 5.0 ng ml^{-1}) and a detection limit of 0.6 ng ml^{-1}. The method was applied to determination of TRFLX in human urine and serum samples. It was validated using HPLC as a reference method. Recovery levels of the method reached 100% in all cases
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Keywords: Differential-pulse adsorptive stripping voltammetry; Trovafloxacin; Urine and serum samples

1. Introduction

Fluoroquinolones are important antibacterials developed in recent years, which have wide applications in veterinary and human medicine.

Trovafloxacin (TRFLX) {(1α, 5α, 6α)-7-(6-amino-3-azabicyclo [3.1.0] hex-3-yl)-1-(2,4-difluorophenyl)-6-fluoro-1,4-dihydroxy-4-oxo-1,8-naphtyridine-3-carboxylic acid} (Fig. 1) is a new synthetic antibacterial fluoroquinolone agent which exhibits high activity against a broad spectrum of Gram-negative and Gram-positive bacteria (aerobic and anaerobic) through inhibition of their DNA gyrase [1].

TRFLX is administered to patients with urinary, respiratory or cutaneous infections in 200 mg per day doses. This drug has a low urine excretion rate, below 5% (in unaltered form) of the administered dosage [1]. Final concentrations in urine and serum of treated patients are in the range 10–15 and 2.2–4.4 μg ml^{-1}, respectively [2].

The widespread use of this compound and the need for clinical and pharmacological study require fast and sensitive analytical techniques to determine the drug in several biological fluids.

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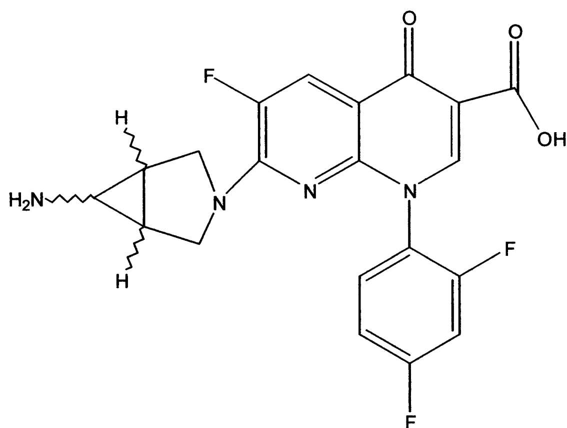

Fig. 1. Structure of TRFLX.

Up to now the most common techniques for the determination of the drug in biological fluids have been based on HPLC with UV detection [3,4] or with fluorimetric detection [5] methods. Recently, a terbium-sensitised luminescence method [6] and a solid-phase spectrofluorimetric method [7] have been proposed for its determination in human serum and urine, respectively.

We are seeking new analytical procedures for the determination of TRFLX alternative to the HPLC and luminescence methods.

Stripping voltammetry (SV) comprises a variety of electrochemical approaches, having a step of preconcentration onto the electrode surface prior to the voltammetric measurement. For the trace analysis of inorganic and organic compounds that cannot be accumulated by electrolysis, the stripping method proposed has been the adsorptive stripping voltammetry (AdSV). In AdSV, the analyte is adsorbed on the working electrode by means of a non-electrolytic process prior to the voltammetric scan [8]. The high sensitivity of adsorptive stripping methods makes it possible to work with very diluted samples with a corresponding decrease in possible interferences in the analysis.

In this paper, a differential-pulse adsorptive stripping voltammetric method for the determination of TRFLX is proposed. The method has been applied to the determination of TRFLX in human urine and serum samples.

The proposed method is the most sensitive reported to date and requires minor analysis total time than HPLC and solid-phase spectrofluorimetric methods because only requires a simple earlier sample treatment.

2. Experimental

2.1. Reagents

All reagents were of the analytical-reagent grade unless stated otherwise. Water was purified with a Milli-Q plus system (Millipore).

Stock solution (0.1 mg ml^{-1}) of TRFLX was prepared by exact weighing of TRFLX mesylate, kindly provided by Pfizer, and dissolution in deionised water. The solution was stable for at least 1 week if stored in the dark at 4 °C. Working solutions were prepared daily by appropriate dilutions with deionised water.

Britton–Robinson buffer solution of pH 4.5 used as supporting electrolyte was prepared in the usual way, i.e. by adding 0.2 M acetic acid (Merck) and 0.2 M boric acid (Merck) to a solution 0.2 M in orthophosphoric acid (Merck), with the appropriated amount of 0.2 M sodium hydroxide (Merck) solution.

2.2. Apparatus and software

Adsorptive and voltammetric experiments were performed using an Autolab (Eco Chemie BV) PGSTST10 potentiostat/galvanostat in conjunction with a Metrohm 663 V stand. A three-electrode system was composed of a static mercury dropping electrode (SMDE), Ag/AgCl reference electrode and a glassy carbon auxiliary electrode.

PGSTAT10 potentiostat/galvanostat was interfaced with an ADL Pentium MMX 200 microcomputer supplied with General Purpose Electrochemical System (GPES) software (Eco Chemie BV) for data acquisition and its subsequent analysis.

All pH measurements were made with an Ingold combined glass-saturated calomel electrode using an earlier calibrated Crison 2000 digital pH-meter.

STATGRAPHICS [9] and ALAMIN [10] software packages were used for the statistical treatment of the data and regression analysis (linear model) and statistical treatment of data.

2.3. Sample treatment

Blank urine samples were obtained from healthy male volunteers and the serum samples were supplied by the 'Virgen de las Nieves' Hospital (Granada).

Real urine samples were obtained from healthy volunteers who received a single oral dose of 200 mg of TRFLX. The samples of individuals were collected for up to 24 h after administration of TRFLX and the urinary volumes were recorded as well.

Urine samples were centrifuged for 10 min at 3800 rpm and filtered through a Minisart-plus syringe filter (0.2 μm pore size, Supelco). Serum samples were filtered through a Centricon 3 centrifugal filter (Amicon) at 7000 rpm.

The filtrates were collected in glass containers that had been carefully cleaned with nitric acid and washed with deionised water and stored at 4 °C until analysis was performed with the minimum possible delay. Aliquots of these filtrates were taken and treated as described in Section 2.4.

2.4. Analytical procedure

To an aliquot of the sample containing between 50 and 500 ng or between 225 and 2000 ng of TRFLX, 5 ml of 0.2 M Britton–Robinson buffer solution (pH 4.5) were added and the solution was diluted with deionised water to 25-ml in a calibrated flask. The solution was first de-aerated by passage of a stream of nitrogen for 10 min. An accumulation potential of −0.30 V was then applied to a fresh drop of mercury, while the solution was stirred at 2500 rpm throughout and accumulation time of 120 s for low linear concentration ranges and 30 s for the highest linear concentration ranges. When the accumulation time was completed, the stirring was stopped and, after a 30 s rest period, a differential pulse scan, with a scan rate of 5 mV s^{-1} and a 50 mV pulse amplitude, was registered from −1.20 to −1.65 V.

A 0.04 M Britton–Robinson buffer solution (pH 4.5), treated in the same way as the sample, was used as blank solution.

The calibration graphs were constructed in the same way with TRFLX solutions of known concentrations.

3. Results and discussion

3.1. Cyclic voltammetry

Fig. 2 shows two sets of sequential cyclic voltammograms for 400 ng ml^{-1} TRFLX in 0.04 M Britton–Robinson buffer solution (pH 4.5). When scanning the potential from −1.00 to −1.65 V (vs. Ag/AgCl) without any accumulation time a no-well defined cathodic peak was obtained because of reduction of dissolved TRFLX, and no peak was observed on the anodic branch, indicating that the reduction of TRFLX is irreversible. When accumulation at −0.30 V was carried out a significantly large adsorptive stripping peak is observed as a result of the adsorption of the initial compound onto the mercury drop (cathodic peak at about −1.53 V).

The spontaneous adsorption of TRFLX can be used as an effective preconcentration step prior to

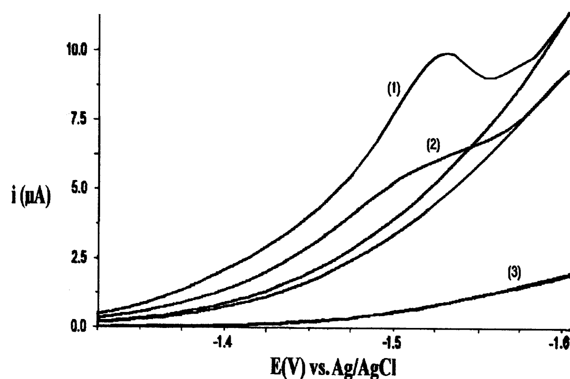

Fig. 2. Cyclic voltammograms of TRFLX: (1) accumulation time of 15 s; (2) without accumulation time. (3) Cyclic voltammograms of no-spiked serum (100 μl) with and without 30 s accumulation time. [TRFLX] = 400 ng ml^{-1}; pH 4.5 (Britton–Robinson buffer); accumulation potential = −0.3 V.

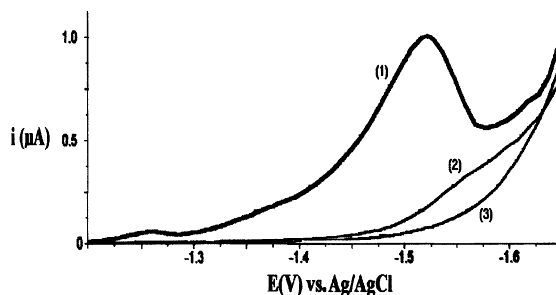

Fig. 3. Differential-pulse voltammograms of: (1) TRFLX with an accumulation time of 30 s. (2) TRFLX without accumulation time (3) blank. [TRFLX] = 100 ng ml^{-1}; pH 4.5 (Britton–Robinson buffer); accumulation potential = −0.3 V.

the voltammetric quantitation of the drug. Fig. 3 shows voltammograms for 100 ng ml^{-1} of TRFLX in 0.04 M Britton–Robinson buffer solution (pH 4.5) and supporting electrolyte only. Although quantitation at this level is not feasible without preconcentration, a well-defined peak was observed following preconcentration at −0.30 V for 30 s.

3.2. pH dependence

The influence of pH on the TRFLX reduction process was studied. The i_p versus pH plot (Fig. 4) shows that the peak current is maximum in the pH interval 4.0–5.0. Different buffer solutions (acetate, monochloroacetate, phosphate and Britton–Robinson) were tested. Britton–Robinson buffer solution (pH 4.5) was found to be the most successful. A 0.04 M concentration of the buffer was selected to obtain an adequate buffering capacity.

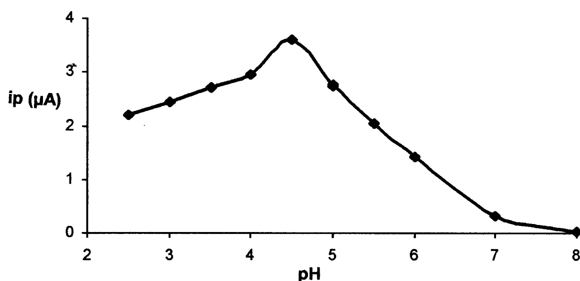

Fig. 4. Influence of pH on peak current (i_p).

3.3. Effect of accumulation potential

The dependence of stripping peak current on the accumulation potential was evaluated over the range −0.10 to −1.00 V for 10 ng ml^{-1} of TRFLX at pH 4.5 for an accumulation period of 120 s. The results obtained shown that the i_p values are maxima for an accumulation potential of −0.30 V.

3.4. Effect of accumulation time

The dependence of stripping peak currents on accumulation time was studied at two concentration levels of TRFLX: 40 and 6 ng ml^{-1}. Taking into account the results obtained, an accumulation time of 30 and 120 s, corresponding to the maximum i_p valued obtained at each concentration level studied, were chosen to evaluated the analytical parameters of the proposed method.

3.5. Instrumental parameters

Several instrumental parameters, such as drop size, stirring rate, scan rate and pulse amplitude, which directly affect to voltammetric response were optimised. The chosen working conditions were: a drop size of 3 (drop area ca. 0.52 mm^2), a stirring rate of 2500 rpm, a scan rate of 5 mV s^{-1} and a pulse amplitude of 50 mV. The stripping currents were not modified when varying the rest period. The chosen value (30 s) is sufficient to allow the formation of a uniform concentration of the analyte in the mercury drop.

3.6. Analytical parameters

Two calibration graphs for the samples treated according to the procedure described above, were constructed. With 120 s accumulation time the calibration graph is linear for the concentration range 2.0–20.0 ng ml^{-1} of TRFLX, and 30 s accumulation time the calibration graph is linear for the concentration range 9–80 ng ml^{-1} of TRFLX.

The lack-of-fit test [11] was used to check the linearity of the calibration graphs. Six replicates

were used for each of five standards prepared to obtain the calibration graphs.

The IUPAC detection limits [12] found were 0.6 and 3 ng ml^{-1} and the quantification limits were 2 and 9 ng ml^{-1}.

The repeatability of the proposed method was determined. The precision was measured for a TRFLX concentration of 5, 10 and 40. ng ml^{-1} by performing ten independent determinations. The relative standard deviations (R.S.D.) were 3.6, 1.2 and 0.9%, respectively.

The statistical and analytical parameters for two calibration graphs are summarised in Table 1.

The proposed method is compared in Table 2 with the methods described to date in literature for the determination of TRFLX. The reported data show an improvement of about two-order of magnitude versus HPLC methods.

3.7. Effect of foreign species

To evaluate de potential effect of foreign ionic species commonly found in urine and serum on the determination of TRFLX at 40 ng ml^{-1} level, a systematic study was carried out. A 20 μg ml^{-1} level of potentially interfering species was tested first and if interference occurred the ratio was reduced progressively until interference ceased. Tolerance was defined as the amount of foreign species that produces an error not exceeding ±5% in the determination of the analyte. Table 3 shows the results obtained.

Table 1
Statistical and analytical parameters

Parameter	Calibration 1	Calibration 2
Intercept (a) (nA)	−3.64	4.85
Intercept standard deviation (s_a)	1.23	0.49
Slope (b) (nA ml ng^{-1})	17.50	0.75
Slope standard deviation (s_b)	0.19	0.01
Correlation coefficient	0.999	0.998
Regression standard deviation (s_{yx})	5.12	0.89
Lack-of-fit test (P-value)	0.36	0.29
Linear dynamic range (ng ml^{-1})	2.0–20.0	9–80
Linearity [1−R.S.D.(b)] (%)	98.9	98.2
Detection limit (ng ml^{-1})	0.6	3
Quantification limit (ng ml^{-1})	2	9

Table 2
Methods for the determination of TRFLX

Technique	Application	Detection limit (ng ml^{-1})	Reference
HPLC-UV	Serum and urine	100	[3]
	Serum	100	[4]
HPLC-FD	Serum and urine	20	[5]
SL	Serum	8	[6]
SPF	Urine	2	[7]
AdSV	Serum and urine	0.6	This work

HPLC-UV: high-performance liquid chromatography-ultraviolet detection; HPLC-FD: high-performance liquid chromatography-fluorimetric detection; SL: terbium-sensitised luminescence; SPF: solid-phase spectrofluorimetry; AdSV: differential-pulse adsorptive stripping voltammetry.

3.8. Application and validation of the method

Table 3
Effect of foreign species on the determination of 40 ng ml^{-1} of TRFLX

Foreign species	Tolerance level (ng ml^{-1})
Cl^-, Na(I), K(I)	> 20 000
Ca(II), Mg(II)	3000
Al(III)	2500
Zn(II)	1000
Cu(II)	500
Fe(III)	100

3.8.1. Spiked samples

The proposed method was applied to the determination of TRFLX in spiked human urine and serum samples using the standard addition method.

The human urine and serum samples were spiked at different levels: 5, 10, 15 and 20 μg ml^{-1} for urine samples and 1, 3 and 5 μg ml^{-1} for serum samples, respectively. The volume range of urine sample used was between 40 and 20 μl and the volume range of serum was between 100 and 50 μl.

Fig. 2 shows cyclic voltammograms of no-spiked serum (100 μl) with an accumulation time of 30 s and without accumulation time. From this figure, it is deduced that serum proteins do not competitively adsorb on the static mercury drop.

The validation of the proposed method for these samples was tested by using a recovery test (Student's t-test) [13,14]. Since the P-values calculated, 36.2% for urine-1, 97.2% for urine-2, 42.3% for serum-1 and 32.1% for serum-2, are greater than 5%, so the null hypothesis appears to be valid, i.e. recovery is close to 100% (Tables 4 and 5).

Table 4
Results of recovery assays to check the accuracy of the proposed method for human urine samples

Sample	Spiked ($\mu g\ ml^{-1}$)[a]	Found ($\mu g\ ml^{-1}$)	Recovery (%)
Human urine-1	5	4.89	97.8
	5	5.22	104.2
	5	5.13	102.6
	5	4.93	98.6
	10	10.13	101.3
	10	9.93	99.3
	10	10.23	102.3
	10	9.83	98.3
	15	15.03	100.2
	15	14.73	98.2
	15	15.01	100.1
	15	15.23	101.5
	20	19.83	99.2
	20	20.03	100.2
	20	20.33	101.7
	20	19.63	98.2
Human urine-2	5	4.83	96.6
	5	4.91	98.2
	5	5.11	102.2
	5	5.22	104.4
	10	9.60	96.0
	10	10.40	104.0
	10	9.77	97.7
	10	10.01	100.1
	15	15.43	102.9
	15	14.58	97.2
	15	14.67	97.8
	15	15.28	101.9
	20	20.46	102.3
	20	19.66	98.3
	20	20.32	101.6
	20	19.74	98.7

Using the Student's t-test: $R = 100.23\%$; $s_R = 0.42$; $t(R) = 1.07$ ($P = 36.2\%$); critical value, 3.182 (5%) for human urine-1. $R = 99.99\%$; $s_R = 0.39$; $t(R) = 0.03$ ($P = 97.2\%$); critical value, 3.182 (5%) for human urine-2.
[a] Referred to original sample.

Table 5
Results of recovery assays to check the accuracy of the proposed method for human serum samples

Sample	Spiked ($\mu g\ ml^{-1}$)[a]	Found ($\mu g\ ml^{-1}$)	Recovery (%)
Human serum-1	1	0.99	99.0
	1	1.01	101.0
	1	0.98	98.0
	1	1.02	102.0
	3	3.04	101.4
	3	2.94	98.0
	3	2.95	98.3
	3	3.07	102.3
	5	4.92	98.4
	5	4.97	99.4
	5	4.98	99.6
	5	5.12	102.4
Human serum-2	1	0.98	98.0
	1	1.00	100.0
	1	1.03	103.0
	1	0.96	96.0
	3	2.95	98.0
	3	3.03	101.0
	3	2.98	99.3
	3	3.05	101.7
	5	5.06	101.2
	5	4.92	98.4
	5	5.03	100.6
	5	4.96	99.2

Using the Student's t-test: $R = 99.98\%$; $s_R = 0.03$; $t(R) = 1.00$ ($P = 42.3\%$); critical value, 3.182 (5%) for human serum-1. $R = 99.70\%$; $s_R = 0.39$; $t(R) = 1.30$ ($P = 32.1\%$); critical value, 3.182 (5%) for human serum-2.
[a] Referred to original sample.

3.8.2. *Real samples*

The proposed method was applied to determination of TRFLX in human urine samples from healthy volunteers who received a single oral dose of 200 mg of TRFLX. The samples were treated as described in Section 2.3.

In this case, HPLC-UV method proposed by Teng et al. [3] was used as a reference method.

The results obtained, summarised in Table 6, shown that both methods (AdSV and HPLC) yield values within the same range when tested using adequate statistical procedures [15].

Table 6
Determination of TRFLX in human urine samples

Sample	Trovafloxacin found (μg ml^{-1})[a]			
	Proposed method	HPLC method	t	P (%)[b]
HU-1	6.8 ± 0.3	6.7 ± 0.2	0.74	47.7
HU-2	13.2 ± 0.3	13.3 ± 0.2	0.38	70.8
HU-3	10.3 ± 0.3	10.1 ± 0.3	1.05	32.1

Urinary volumes: 1.4 l for HU-1; 1.2 l for HU-2 and 1.5 l for HU-3.
[a] Average value ± S.D. of five determinations.
[b] P value of the two-sample comparison test.

4. Conclusions

A sensitive and practical differential-pulse adsorptive stripping voltammetric method for the determination of the antibacterial TRFLX at ng ml^{-1} level is presented. The detection limit obtained, 0.6 ng ml^{-1}, was the lowest reported up to date. It was applied satisfactorily to human urine and serum samples with good recovery rates in all cases.

This is a rapid one step procedure which only requires a simple earlier sample treatment and to perform its voltammogram, so it is an inexpensive, simple and fast procedure which does not need an earlier separation of the analyte.

Although HPLC and solid-phase spectrofluorimetric methods can be used to determine this drug in human urine and serum samples and usually, they can offer more accuracy than the proposed method, they are both more time consuming and expensive than the procedure here developed.

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