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Design and optimization of the variables in the adsorptive stripping voltammetric determination of rufloxacin in tablets, human plasma and urine*

S. FURLANETTO,†‡ P. GRATTERI,‡ S. PINZAUTI,‡ R. LEARDI,§ E. DREASSI‖ and G. SANTONI¶

‡ *Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via G. Capponi 9, 50121 Firenze, Italy*
§ *Istituto di Analisi e Tecnologie Farmaceutiche ed Alimentari, Università di Genova, Via Brigata Salerno (ponte), 16147 Genova, Italy*
‖ *Dipartimento Farmaco Chimico Tecnologico, Università di Siena, Banchi di Sotto 55, 53100 Siena, Italy*
¶ *Stabilimento Chimico Farmaceutico Militare, Via R. Giuliani 201, 50141 Firenze, Italy*

Abstract: An adsorptive stripping voltammetric method with a hanging mercury drop electrode was developed for the determination of the fluoroquinolone rufloxacin in tablets, human plasma and urine. Measurements were obtained in differential pulse mode and a multivariate strategy was used to optimize the variables involved. Besides the independent effects of the variables, a strong interaction between scan rate and pulse duration has been found. Rufloxacin was analysed at concentrations between 1.7×10^{-8} and 1.9×10^{-7} M with a detection limit of 9.2×10^{-9} M. Diluted tablet solutions and urine samples were analysed directly, while plasma samples needed an extraction procedure before voltammetric analysis. An improved HPLC procedure was used as comparative method.

Keywords: *Adsorptive stripping voltammetry; rufloxacin; human plasma and urine analysis; experimental design; regression modelling; response surface plot; HPLC determination.*

Introduction

Rufloxacin is a long-acting new tricyclic fluorinated quinolone (Fig. 1). This group of drugs is bactericidal over a wide range of therapeutically-achievable concentrations and act via selective inhibition of bacterial DNA synthesis. Rufloxacin has a broad range of action against Gram-positive and Gram-negative microorganisms, including β-lactamase resistant ones.

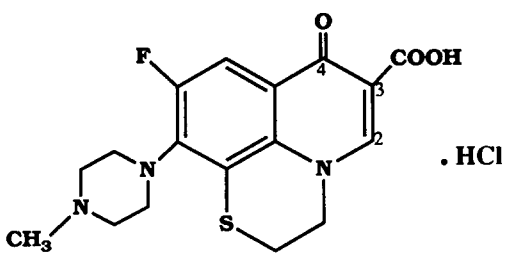

Figure 1
Structure of rufloxacin.

Preliminary information on the single-dose pharmacokinetics of rufloxacin indicates that the maximum serum level following a 400 mg oral dose is 3–6 μg ml^{-1} with slow elimination and a plasma half-life of 12–24 h, thus suggesting the valid clinical use of the drug in the treatment of urinary and respiratory infections, bacterial gastroenteritis and gonococcal disease [1]. Thus in long-term treatment 200 mg oral doses of rufloxacin, preceded by a loading dose of 400 mg, are needed to establish a mean plasma concentration of 4.51 μg ml^{-1}. The urinary concentration corresponding to the same dosage regime was 29.75 μg ml^{-1} between 48 and 72 h after the final administration [2].

Up to now the most commonly-employed techniques for the determination of the drug and its metabolites in biological fluids have been based on HPLC [3–7] but UV-spectroscopy [8] and fluorimetry [9] have also been used. However, the presence of both the reducible keto-group C=O on C_4 and the double-bond between C_2–C_3 make rufloxacin

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†Author to whom correspondence should be addressed.

an interesting candidate for an electroanalytical method of analysis. The present paper describes the development of a highly sensitive procedure based on adsorptive stripping voltammetry (AdSV) which is able to decrease the limit of quantitation previously reported for other techniques. It also aims to allow analysis at therapeutic levels in biofluids of patients undergoing treatment. Moreover, an improved HPLC procedure was set up and used as a comparative method for the proposed voltammetric procedure.

Experimental

Materials

Lomefloxacin hydrochloride, rufloxacin hydrochloride and pipemidic acid bulk materials and rufloxacin tablets (Qari®) were kindly supplied by Mediolanum (Milan, Italy). Methanol and methylene chloride were HPLC grade and obtained from E. Merck (Darmstad, Germany). All solutions were prepared using water obtained by passing deionized water through a Milli-Q water purification system. The 0.01 M potassium chloride solution, used as supporting electrolyte, and 0.2 M sodium hydroxide solution were prepared from Suprapur® quality reagents (E. Merck, Darmstadt, Germany). Analytical reagent-grade sodium heptane sulphonate and tetramethylammonium chloride (TMA) were obtained from BDH (Poole, England). Phosphate buffer at pH 2.7 was prepared by adjusting the pH of a 0.025 M monopotassium phosphate solution with 0.04 M orthophosphoric acid solution. Phosphate buffer at pH 7 was prepared by adding to 100 ml of a 0.1 M bisodium phosphate solution a suitable amount of 0.1 M monopotassium phosphate solution to reach the desired pH. The human urine samples were collected daily from at least five healthy individuals and a pool of these was used. Plasma samples were obtained from the Banca Militare del Sangue (Florence, Italy).

Apparatus

A voltammetric system, consisting of an AMEL 433 Polarographic Analyser incorporating a mercury electrode (DME or HMDE) as working electrode, saturated silver/silver chloride as the reference and a platinum wire as auxiliary electrodes, was used. A magnetic stirrer incorporated into the polarographic analyser and a stir bar provided the convective transport during the preconcentration. The experiments were carried out at room temperature in an all-glass cell. Data handling, storage, printout and graphics were controlled by means of a host computer and special AMEL software. All pH measurements were made with a Metrohm 691 pH meter. A Hattich Universal 30F centrifugal separator, a Janke & Hunkel Ika-Vibrax-VXR vortex and a Ney Ultrasonic 300 sonicator were used. The HPLC system consisted of a Perkin–Elmer model LC 620 pump equipped with a Perkin–Elmer model ISS 200 autosampler and a Perkin–Elmer model LC 235 UV diode-array detector. Data handling, storage, printout and graphics were controlled by means of a host computer equipped with Perkin–Elmer LC Analyst software. The analytical separation was achieved on a Waters Novapack C_{18} column (15 cm × 3.9 mm i.d., 4-μm particle size). A RCSS C_{18} guard-PAK was placed just before the inlet of the analytical column.

The response surface modelling and the multivariate regression calculation were supported by the package "NEMROD", version 2.01 (LPRAI, Université de Marseille III, Marseille-Le Merlan, France).

Voltammetry

Operation conditions. The investigations were performed by de-aerating 10 ml of the supporting electrolyte with highly purified nitrogen for 400 s in the initial cycle, and then for 30 s in each successive cycle, followed by applying the optimized experimental conditions found by experimental design: accumulation potential, −700 mV; accumulation time with stirring, 60 s; stirring speed, 600 rpm; drop size, 30 arbitrary units (a.u.); scan rate, 40 mV s^{-1}; pulse amplitude, 60 mV; rest time after accumulation, 10 s; pulse width, 20 ms. Scans were performed from −1200 to −1600 mV vs Ag/AgCl.

Calibration procedure. Fresh stock solution of rufloxacin (0.15 mg ml^{-1}) was prepared by dissolving 15 mg of the drug in 100 ml of water. A diluted working solution (1.5 μg ml^{-1}), used within 3 days, was prepared by making up 0.5 ml of fresh stock solution to 50 ml with water. The voltammetric studies were performed by placing 10 ml of the supporting electrolyte into the polarographic cell; the solution was deoxygenated in the first cycle for 400 s and in each successive cycle for

30 s with a stream of pure nitrogen and the nitrogen atmosphere was maintained throughout the experiments. The optimized experimental conditions found by experimental design were applied and the voltammogram of the supporting electrolyte was recorded. A calibration graph was prepared by first recording a voltammogram of the blank and by subsequently micropipetting six aliquots of the working solution (40 μl) into the polarographic cell. Each voltammogram was recorded twice using a new mercury drop. The calibration plots for plasma and urine were obtained using an appropriate blank consisting of 10 ml of 0.01 M potassium chloride, to which 30 and 150 μl of unspiked plasma and urine solution were added, respectively. Seven additions (40 μl) of the working solution were then made. The calibration graphs were constructed by plotting the peak current against the corresponding concentration. Quantification was achieved by means of the calibration curve method.

Tablet analysis. Twenty tablets which contained a declared amount of rufloxacin (200 mg) were selected at random, accurately weighed and the average value was calculated. The tablets were crushed and an accurately weighed portion of the powder, equivalent to about 15 mg of rufloxacin, was transferred into a 100-ml calibrated flask, dispersed in 50 ml of water and sonicated for 5 min to ensure complete dissolution of the drug. The resulting suspension was diluted to volume with water. A 1 ml aliquot of this solution was made up to 25 ml with water and the resulting solution (6.25 μg ml^{-1}) was used for the voltammetric analysis. Quantification of the drug was carried out using the regression equation relating the peak current (resulting from the addition of 50 μl of rufloxacin tablet solution into the cell containing 10 ml of the supporting electrolyte) to concentration.

Urine analysis. A 1.5-ml aliquot of rufloxacin solution (1 mg ml^{-1}) was made up to 50 ml with drug-free urine to obtain a urinary concentration of 30 μg ml^{-1} [2]. A 1 ml aliquot of the spiked urine sample was made up to 20 ml with water and the resulting rufloxacin-urine solution (1.5 μg ml^{-1}) was used for the voltammetric investigations. Unspiked urine samples were obtained in the same way. The analysis was carried out by adding 150 μl of rufloxacin–urine solution to the cell containing 10 ml of 0.01 M potassium chloride, and recording the voltammogram. Quantification was performed by means of the calibration curve method.

Plasma analysis. One millilitre of plasma was transferred into a Teflon centrifuge tube, spiked with 30 μl of the rufloxacin solution (0.15 mg ml^{-1}) in order to obtain a suitable human plasma concentration [2], and added to 2 ml of acetonitrile. The mixture was vortexed for 3 min and centrifuged for 3 min at 2800*g*. The protein precipitate was discarded and the supernatant solution was added to 4 ml of methylene chloride in another centrifuge tube. The latter was vortexed for 5 min and centrifuged for 3 min at 2800*g*, then the organic phase was transferred to a separate tube. A 4-ml aliquot of fresh methylene chloride was added to the first tube and the same extraction procedure repeated. The organic phases collected from the two extractions of the same sample were pooled and evaporated under a stream of pure nitrogen. The residue was reconstituted with 0.5 ml of water and the solution was used for voltammetric study. Unspiked plasma solution was obtained in the same way. Quantification was performed by means of the calibration curve method.

HPLC method

Calibration curves. Appropriate amounts of rufloxacin and of the internal standard lomefloxacin were dissolved in 1 ml of 0.1 M NaOH and then diluted to 50 and 100 ml with water, respectively, thus obtaining a 25 μg ml^{-1} rufloxacin stock solution and a 50 μg ml^{-1} lomefloxacin stock solution. The concentrations of the rufloxacin working standard solutions, used to construct the calibration curve for tablet analysis and prepared by appropriate dilution of the stock solution with water, ranged from 5 to 15 μg ml^{-1}. The concentration of lomefloxacin, used as internal standard, was fixed at 10 μg ml^{-1}.

Spiked plasma working solutions were obtained by treating in parallel four 1 ml aliquots of plasma with 2 ml of 0.1 M phosphate buffer (pH 7) and the appropriate amount of rufloxacin standard solution (4 μg ml^{-1}), thus obtaining solutions with concentrations ranging from 0.4 to 1.2 μg ml^{-1}. To these solutions 4 ml of methylene chloride were added and the mixtures were vortexed for

10 min. Separation of the two phases was achieved by centrifuging at 3000*g* for 1 min, sonicating for 10 min and centrifuging again at 3000*g* for 10 min. The organic phase was collected in a second tube while 4 ml of fresh methylene chloride were added to the first tube and the same extraction procedure repeated twice. The organic phases deriving from the three extractions of the same sample were pooled and evaporated under a stream of pure nitrogen. The residue was reconstituted with 0.5 ml of a 0.05 M sodium hydroxide pipemidic acid solution (1 $\mu g\ ml^{-1}$) used as internal standard. The solutions were used for the construction of the calibration curve for plasma analysis. The same procedure was applied to solutions prepared using 1 ml of water instead of 1 ml of plasma and used as reference standards. A 50-μl volume of each solution was injected in triplicate.

Four 1 ml aliquots of urine were treated in parallel with 1 ml of 0.05 M sodium hydroxide pipemidic acid solution (0.1 $\mu g\ ml^{-1}$) used as internal standard and with an appropriate amount of rufloxacin standard solution (0.1 mg ml^{-1}). The solutions were made up to 50 ml with water, thus obtaining rufloxacin concentrations ranging from 1 to 2.5 $\mu g\ ml^{-1}$ with a fixed concentration of the internal standard pipemidic acid (2 $\mu g\ ml^{-1}$). These solutions were used to construct the calibration curve for urine analysis. A 20-μl volume of each solution was injected in triplicate.

Calibration curves for each sample were obtained by plotting the peak–height ratios between rufloxacin and the internal standard vs the corresponding analyte concentrations.

Tablet analysis. Twenty tablets which contained a declared amount of rufloxacin of 200 mg were selected at random, accurately-weighed and the average weight calculated. The tablets were crushed and four accurately weighed portions of the powder, equivalent to about 200 mg of rufloxacin, were transferred into 100-ml calibrated flasks, made up to volume with 0.1 M sodium hydroxide and the resulting suspensions filtered (0.45 μm). Aliquots of 5 ml were diluted to 100 ml with water. From the latter solutions, aliquots of 1 ml were transferred into 10-ml volumetric flasks, 2 ml of the internal standard solution added and the flasks then made up to volume with water. A 25-μl aliquot of each solution was injected in triplicate. The mobile phase comprised: phosphate buffer (pH 2.7; 0.025 M)–methanol–TMA (100 mM)–sodium heptane sulphonate (100 mM) (46:42:2:10, v/v/v/v). The flow rate was 1 ml min^{-1}.

Plasma and urine analysis. There were no endogenous peaks which coeluted with rufloxacin. The mobile phase comprised: methanol–TMAC (100 mM)–phosphate buffer (pH 2.7; 0.025 M) (15:2:83, v/v/v). The flow rate was 1.2 ml min^{-1}. The solutions used for analysis were prepared as described above for the construction of the calibration curve.

Results and Discussion

The setting up of a voltammetric method of analysis requires consideration of a great number of variables, which can differ according to the technique under study. The use of adsorptive stripping voltammetry applied to the assay of rufloxacin, involves accumulation time and accumulation potential in addition to scan rate, scan mode, temperature, type of background electrolyte and its characteristics such as ionic strength, and so on. These must all be carefully optimized in order to find the best value to attribute to each variable and obtain the best result. Up to now the most common method for optimizing the experimental conditions of the whole procedure is to consider "one factor at a time", although such approach does not take into account the possible interdependence between variables and, in most cases, does not allow one to select which variables most influence the response.

The authors' previous efforts [10] have shown how the use of multivariate methods can also be usefully applied to voltammetric analysis, allowing all pertinent factors to be considered simultaneously, both for designing experiments and for analysing the results, thus avoiding the need to carry out useless and time-consuming experiments. Also in the present work the optimization was performed stepwise, starting from a large number of variables, each with a rather wide range, and postulating very simple models. At each step some of the variables were eliminated, the range of the remaining variables was adjusted and the postulated model was adapted.

Two responses were taken into account: peak height and the reproducibility of results, expressed as 'variation coefficient' computed on the basis of two independent experiments.

Screening design

The preliminary investigations carried out on the drug indicated that potassium chloride is an appropriate supporting electrolyte, thus allowing this variable to be eliminated from the initial screening. Seven factors were considered as possibly influencing the outcome of the experiments. The first step of the optimization procedure was an eight-way Plackett–Burman design [11], assuming, at this point of the study, that a linear model without interactions would be satisfactory. The factors considered were: ionic strength, accumulation time, accumulation potential, scan rate, stirring rate, drop size and pulse amplitude. Each of these was tested at two different levels (high and low) (Table 1).

Regarding peak height, all factors, except for drop size, were found to be significant and all the coefficients had a positive sign, except for ionic strength, thus indicating that the higher value should be preferred.

As regards reproducibility, no factors were found to be significant; as a consequence, in the following step only peak height is considered. Had this not been the case, then a simultaneous optimization of the two responses should have been performed, in an attempt to find a compromise solution producing the highest peak with acceptable reproducibility.

As can be seen, experimental design is a general approach, which can be applied to whatever feature it is desired to optimize; another important aspect is the possibility of dealing with several responses at a time.

D-optimal design

In the second step of the optimization process, the stirring rate was shown not to be significant after this screening and was eliminated. Since in the first step its coefficient was slightly positive, it was decided to keep it constant at the high level (600 rpm). Although drop size was not significant in this case and since it was thought that it could anyway have some influence on the process; it was decided to keep it in the following step and a new factor, pulse duration, was introduced.

The authors' earlier work [10] had shown that interaction between factors might be extremely important, so a model was postulated which took into account the principal effects and the first-order interactions. With seven factors, this model had 29 coefficients (one constant term, seven principal effects and 21 interactions). The variables and their levels are reported in Table 2. In this step it was decided to perform a maximum of 32 experiments, i.e. the number of experiments which could be performed in a single day. The experiments were selected by a D-optimal

Table 1
Variables and experimental domain for the Plackett–Burman design

$$y = \beta_0 + \Sigma\beta_i x_i + e$$

Variable	Low level (−)	High level (+)
Ionic strength (M)	0.01	0.1
t_{acc} (s)	40	90
E_{acc} (mV)	−200	−700
Scan rate (mV s^{-1})	10	30
Drop size (a.u.)	10	30
Stirring rate (rpm)	200	600
Pulse amplitude (mV)	30	70

Table 2
Variables and experimental domain for the D-optimal design

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\Sigma\beta_{ij} x_i x_j + e \text{ (where } i \neq j)$$

Variable	Low level (−)	High level (+)
Ionic strength (M)	0.01	0.05
t_{acc} (s)	40	70
E_{acc} (mV)	−400	−700
Scan rate (mV s^{-1})	20	40
Drop size (a.u.)	10	30
Pulse duration (ms)	20	40
Pulse amplitude (mV)	50	70

design having as candidate points the 128 points deriving from a 2^7 full factorial design. This technique makes it possible to detect the best subset of experiments from a set of candidate points; the analysis of several parameters (determinant of the information matrix, inflation factors, leverage) allows one to evaluate the quality of the information which would be obtained after having performed the experiments. When repeated with subsets of different size, it gives a very good idea of the evolution of these parameters with increase in the number of the experiments, so that a good compromise can be found between the quality of the information obained and the number of experiments to be performed. In the present case, the quality of information obtained from the experimental matrix containing 32 experiments was considered sufficient at this stage, so this number was selected since it gave much better information than that obtained with 31 experiments.

The results obtained show that, except for the accumulation potential, all the principal effects are significant and, except for ionic strength and pulse duration, the higher level gives the better results. Of the interactions, only the accumulation time–pulse duration and scan rate–pulse duration had a significance level of <0.10. Following on from this step, it was decided to set the final values for ionic strength (0.01 M), accumulation potential (700 mV) and drop size (30 a.u.) and to investigate the four remaining factors.

Full factorial design

A full factorial design with two central points for a total of 18 experiments, was performed. The levels of the variables were adjusted according to the results of the previous step (Table 3). The significant factors were found to be pulse duration and scan rate (both with $p < 0.05$); their interaction is also at the same significance level. Since no significant difference was found between the central points and the average of the responses, it can be assumed that the postulated model fits the data well in the experimental domain under study, and that no curvature is present.

The effect of interaction can be very easily seen in Fig. 2, where it is evident that the effect of pulse duration is relevant only when the scan rate is at the higher level, and that the effect of scan rate is much higher when the pulse duration is at the lower level. This result gives a clear demonstration of the superiority of the multivariate approach over the one-variable-at-a-time method. In the latter case, in fact, the interaction could not be detected. Since the two interacting variables are already at the limits of their possible values, the optimization can be considered as concluded. The optimal conditions are reported in Table 4.

Calibration curves

Using the optimized conditions, a well-defined reduction peak occurring at −1350 mV (vs Ag/AgCl) was registered and a linear regression plot was established from 1.7×10^{-8} to 1.9×10^{-7} M, yielding the equation: $y = 0.024$ (μA l μg^{-1}) -0.003 (μA). Figure 3 shows the AdSV response obtained by successive addition (40 μl) of rufloxacin working solution. The detection limit was calculated as the analyte concentration giving a signal equal to the blank signal y_B plus two standard deviations of the intercept and was equal to 9.2×10^{-9} M. To check the lack of fit of the model, two estimates s_1^2 and s_2^2, of the error variance were calculated through two different and independent ways:

$$s_1^2 = \Sigma e_i^2/df_1,$$

where e_i = residuals = $y_i^{Obs} - y_i^{Pred}$, and df = degree of freedom and

$$s^2 = (x_i - \bar{x})^2/df_2,$$

Table 3
Variables, experimental domain and first interaction model for the 2^4 full-factorial design

$$y = \beta_0 + \Sigma\beta_i x_i + \Sigma\Sigma\beta_{ij} x_i x_j + e \text{ (where } i \neq j)$$

Variable	Low level (−)	High level (+)
t_{acc} (s)	60	70
Pulse duration (ms)	20	30
Scan rate (mV s^{-1})	30	40
Pulse amplitude (mV)	50	70

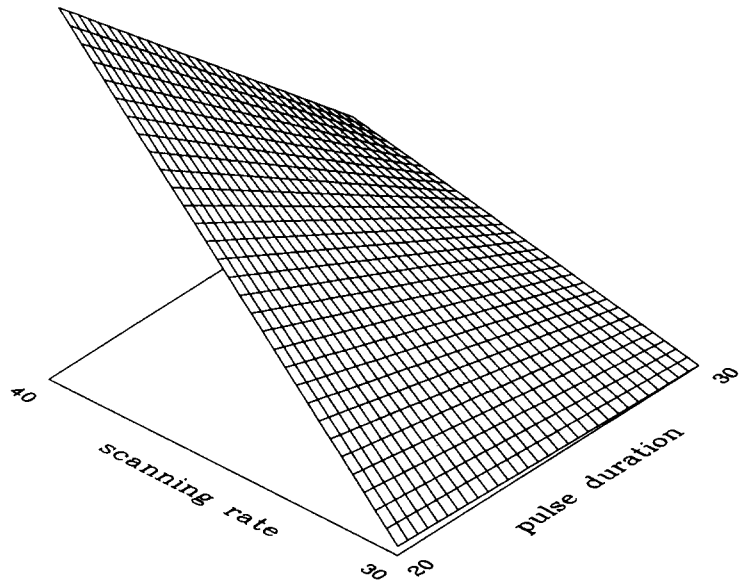

Figure 2
Three-dimensional plot of the response surface in the voltammetric study of rufloxacin. Scan rate (mV s^{-1}) vs pulse duration (ms), the unlabelled *y*-axis represents the peak height response.

Table 4
Optimized experimental conditions

E_{acc} (mV)	t_{acc} (s)	Ionic strength (M)	Pulse duration (ms)	ΔE (mV)	v (mV s^{-1})	Drop size (a.u.)	Stirring rate (rpm)
−700	60	0.01	20	60	20	30	600

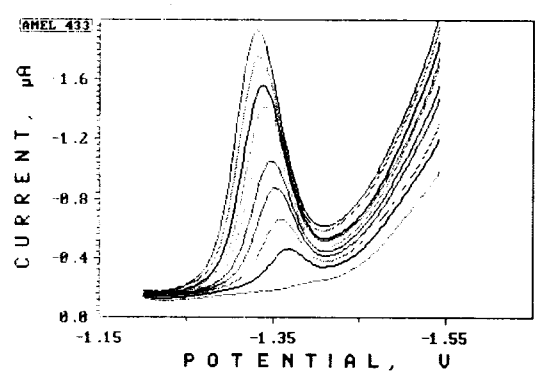

Figure 3
Adsorptive stripping voltammograms of increasing rufloxacin concentration from 0 to 1.46×10^{-7} M obtained by successive additions of 40 µl of rufloxacin standard (1.5 µg ml^{-1}). Optimized conditions: as in Table 4.

where x_i = repeated measurements of the same sample concentration performed on different samples. The *F*-ratio, $F = s_1^2/s_2^2$, with df_1 and df_2, respectively, degrees of freedom, did not exceed the critical *F*-ratio for the significance level $\alpha = 5\%$, thus suggesting an insignificant lack of fit of the model [11]. However the model was also validated by comparing the standard solution mean concentration value ($n = 10$) predicted, with the theoretical concentration of the same solution.

Quantification of rufloxacin in tablets, human plasma and urine was obtained with the calibration curve method. Table 5 reports the calibration data for urine, plasma and tablets using, for the latter, a more restricted range than that described for the linearity range and corresponding to 40–120% of the declared in-cell rufloxacin concentration. The presence of plasma in the supporting electrolyte affected the adsorption behaviour of rufloxacin at the mercury electrode, making the clean-up procedure described above necessary prior to AdSV analysis. The extraction yield, based on the slope ratio, i.e. the ratio between the slope of the line obtained by standard additions to spiked plasma and the slope of the line obtained by standard additions to blank and multiplied by 100, was 83.0%. Contrary to the data with plasma, neither the excipients of the pharmaceutical preparation, nor the naturally-

Table 5
Linear regression parameters for tablet, urine and plasma determination by AdSV

	Slope ($\mu A\ l\ \mu g^{-1}$)	Intercept (μA)	r^2	Linear range ($\mu g\ l^{-1}$)
Tablets	2.54×10^{-2}	-3.87×10^{-2}	1.00	9.50–37.7
Urine	1.90×10^{-2}	5.90×10^{-2}	0.993	6.42–44.0
Plasma	2.22×10^{-2}	2.77×10^{-2}	0.997	5.62–38.6

Table 6
Percentage recoveries of rufloxacin in tablets, urine and plasma obtained with AdSV and HPLC

	AdSV			HPLC		
	Concentration level ($\mu g\ l^{-1}$)	% Recovery	RSD ($n = 5$)	Concentration level ($\mu g\ l^{-1}$)	% Recovery	RSD ($n = 5$)
Tablets	29.5	109.7	4.1	10.1×10^3	112.8	2.3
Urine	23.5	103.3	2.9	1.8×10^3	96.7	2.9
Plasma*	25.5	102.9	1.2	0.8×10^3	105.0	3.3

* Results related to the extraction yield.

occurring components in urine caused interferences during the assay. This was evidently attributable to the high dilution of the solutions assayed deriving from the highly sensitive technique employed and the low concentration of the drug required in the cell.

Comparison of AdSV and HPLC results

The results for the AdSV determination of rufloxacin in tablets, urine and human plasma were compared with those obtained with the above described HPLC method (Table 5). The HPLC plasma recovery was assayed by comparing the extracted plasma sample–internal standard peak height ratio, vs the aqueous standard–internal standard peak height ratio. The observed extraction yield was 89%. As can be seen, the results obtained with the voltammetric method are in good agreement with those of the HPLC procedure. However, the voltammetric method is simpler, faster and more sensitive; moreover, it requires less expensive equipment and less sample preparation handling than does the chromatographic method (Table 6).

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