Optimization Procedure, Applying the Experimental-Design Methodology, for the Determination of Rifampicin after Metal Complexation by Differential Pulse Adsorptive Stripping Voltammetry

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A procedure for the determination of rifampicin (RIF) after Cu^{II} complexation by differential pulse adsorptive stripping voltammetry (DPAdSV) was optimized. The selection of the experimental conditions was made on the basis of the experimental-design methodology. The calibrations were performed under these conditions by means of a robust regression method that allows for the elimination of anomalous points. Once the detection limit was calculated, this method was successfully applied to the analysis of RIF in pharmaceutical preparations. The analysis of urine samples was also performed. The complexity of these samples made it necessary to apply multivariate-regression techniques to obtain satisfactory results.

1. Introduction. – The rifamycin group comprises structurally similar complex macrocyclic antibiotics that constitute the active principle of several pharmaceutical products to treat tuberculosis, leprosy, and other infections. They are natural or semisynthetic products formed from condensed naphthalene and furan rings, connected by an aliphatic bridge [1]. Rifampicin (RIF), the most common compound in the class, inhibits the growth of most *Gram*-positive and some *Gram*-negative microorganisms by acting on DNA-dependent RNA polymerase and forming a stable drug–enzyme complex that results from suppressing the initiation of chain formation in RNA synthesis [2].

Several analytical procedures were reported for the analysis of RIF in pharmaceutical products and biological fluids including HPLC [3–5], thin-layer chromatography [6], spectrophotometry [7][8], and voltammetry [9].

Due to the presence of hydroxy and amide groups in the RIF molecule, complex formation with 'biometals' such as Cu^{II} and Zn^{II} could be expected [10]. Methods to determine ansamycins (rifamycin SV and RIF) by spectrophotometric analysis, based on the effect of Cu^{II} ions, are described [11][12].

In the last few years, electrochemical methods have been developed based on adsorption phenomenon that exhibit numerous organic compounds in some electrodes. This has allowed the application of a considerable number of elements, by procedures that are both conceptually simple and experimentally easy to perform. The improvement in the analytical signal obtained as a result of the preconcentration process allows stripping voltammetry to be considered one of the most accurate and sensitive techniques.

The aim of this research work was to set up a method for RIF determination in an aqueous medium by means of differential pulse adsorptive stripping voltammetry

(DPAdSV) after complexation with Cu^{II} ions, and by application of the univariate calibration methodology. This method was successfully applied to pharmaceutical preparations. However, a soft calibration was necessary, such as partial least-squares (PSL) [13][14], when urine samples were tested, due to the presence of interferences.

The large number of experimental variables that can affect the result when stripping voltammetry techniques are used means that the variables must be optimized to enable measurement under the best conditions [15][16]. An appropriately designed experiment provides signals of far superior quality to those measured in an experiment that has not been optimized. Likewise, the use of experimental designs allows to use a reduced number of experiments to explore a wide experimental range. They are more efficient than the 'one-at-a-time' experiments since they allow detection of interactions between factors that could lead to false conclusions. Therefore, the experimental design was used here to optimize the influencing variables, such as potential and time of deposition, concentration of Cu^{II}, and the pH value.

2. Experimental. – 2.1. *General.* Anal.-grade chemicals were used with no further purification. All the solns. were prepared with *Milli-Q* water. N_2 (99.99%) was used to remove dissolved O_2. Solns. of rifampicin were prepared by dissolving the appropriate amount of rifampicin (*Fluka*, Buchs, Switzerland) in H_2O. Stock standard solns. of Cu^{II} were prepared by dissolving the appropriate amount of $CuSO_4 \cdot 5\,H_2O$ (*Merck*, Darmstadt, Germany) in H_2O. $NaHCO_3$, NaOH (*Merck*, Darmstadt, Germany) buffer was used.

Voltammetric measurements were carried out by means of a *μAutolab* of type *II* (*Eco Chemie*) with a *Metrohm 663-VA* (*Methrohm*, Herisau, Switzerland) electrode stand with a multimode electrode (MME) operating in the hanging-mercury-drop electrode (HMDE) mode. An Ag/AgCl 3M KCl reference electrode and a Pt-wire auxiliary electrode were also used. The pH of the soln. was measured with a *Crison 2002* (Barcelona, Spain) pH meter.

Voltammograms were acquired and processed with the general-purpose electrochemical system software (GPES) [17]. Data analysis was achieved with the STATGRAPHICS PLUS software package [18] for the experimental design, PROGRESS [19] for the robust regression, and PARVUS [20] for the multivariate regression models.

2.2. *Voltammetry.* Voltammetric measurements were made by the following procedure: the soln. was purged and stirred for 300 s, then the deposition potential was applied according to a time and potential determined for each experiment. The soln. was left to rest for an equilibrium time of 5 s, then a cathodic scan from 0 V (initial potential) to -0.8 V (final potential) was started and the voltammogram recorded. Other experimental parameters were the following: mercury-drop size, 0.52 mm^2; stirring rate in the deposition period, 1500 rev. min^{-1}; modulation amplitude, 50 mV; step potential, 6 mV; modulation and interval time, 0.04 s and 0.6 s, resp.

3. Results and Discussion. – 3.1. *Optimization of Experimental Variables. Preamble.* Previous experiments showed that RIF reacts with Cu^{2+} ions giving rise to a complex, stable with time, that has a reduction peak at approximately -0.35 V. The electrochemical reduction of this complex is affected by several experimental variables, such as deposition time and potential, pH, or the concentration of the metal. To obtain a good analytical signal that allows for the determination of RIF, these variables were optimized by means of the experimental-design methodology [15][16].

Initially, a factorial design was carried out, taking into account the four influential variables. The values corresponding to the high (+) and low (−) levels and to the central point (0) for each factor were given in *Eqns. 1–3*. Although the result of this design did not lead to acceptable results, it did indicate the direction of experimentation to be taken into account in successive optimization stages. The results are shown below.

$t_{dep}(+) = 70$ s $\quad E_{dep}(+) = 0$ V $\quad C_{Cu}(+) = 1 \cdot 10^{-5}$ M $\quad$ pH(+) = 11 (1)

$t_{dep}(-) = 20$ s $\quad E_{dep}(-) = -0.8$ V $\quad C_{Cu}(-) = 1 \cdot 10^{-6}$ M $\quad$ pH(−) = 9 (2)

$t_{dep}(0) = 45$ s $\quad E_{dep}(0) = -0.4$ V $\quad C_{Cu}(0) = 5.5 \cdot 10^{-6}$ M $\quad$ pH(0) = 10 (3)

First Stage: 2^4 Factorial Design. This stage of the optimization process again consisted of a 2^4 factorial design. Two levels were selected, high and low, for each of the factors. Then 16 experiments were carried out corresponding to all the possible combinations, bearing in mind the three replicates in the central point necessary to estimate the residual value. The values corresponding to the high (+) and low (−) levels and to the central point (0) for each factor were as given in *Eqns. 4–6*.

$t_{dep}(+) = 30$ s $\quad E_{dep}(+) = -0.2$ V $\quad C_{Cu}(+) = 3 \cdot 10^{-6}$ M $\quad$ pH(+) = 11 (4)

$t_{dep}(-) = 14$ s $\quad E_{dep}(-) = -0.6$ V $\quad C_{Cu}(-) = 7 \cdot 10^{-6}$ M $\quad$ pH(−) = 9 (5)

$t_{dep}(0) = 22$ s $\quad E_{dep}(0) = -0.4$ V $\quad C_{Cu}(0) = 5 \cdot 10^{-5}$ M $\quad$ pH(0) = 10 (6)

The results obtained for this experimental design are reported in *Table 1*. The experiments in which no response was obtained were quantified with a value of 0.100 nA to allow analysis of the results. The analysis is shown in the form of ANOVA (*Table 2*). It can be seen that neither the deposition potential nor its interactions influence the value of the response ($P_{actual} > 0.05$), such that this factor was set at −0.4 V, the value corresponding to the central point, and close to the reduction potential of the complex.

The influence of the principle factors on the response can be seen in *Fig. 1*, which indicates the values of the factors that must be considered in the next step. This implies that both deposition time and the pH value must be reduced and the concentration of

Table 1. *Results of the 2^4 Factorial Design for the Optimization of Experimental Parameters in the Formation of the Complex [Cu(RIF)] by DPAdSV.* $C_{RIF} = 4 \cdot 10^{-6}$ M.

t_{dep} [s]	E_{dep} [V]	C_{Cu} [M]	pH	$-I_p$ [nA]
14	−0.6	$3 \cdot 10^{-6}$	9	3.299
30	−0.6	$3 \cdot 10^{-6}$	9	4.828
14	−0.2	$3 \cdot 10^{-6}$	9	4.805
30	−0.2	$3 \cdot 10^{-6}$	9	0.100
14	−0.6	$7 \cdot 10^{-6}$	9	9.972
30	−0.6	$7 \cdot 10^{-6}$	9	5.202
14	−0.2	$7 \cdot 10^{-6}$	9	10.330
30	−0.2	$7 \cdot 10^{-6}$	9	0.100
14	−0.6	$3 \cdot 10^{-6}$	11	1.405
30	−0.6	$3 \cdot 10^{-6}$	11	0.100
14	−0.2	$3 \cdot 10^{-6}$	11	2.386
30	−0.2	$3 \cdot 10^{-6}$	11	0.868
14	−0.6	$7 \cdot 10^{-6}$	11	1.799
30	−0.6	$7 \cdot 10^{-6}$	11	0.100
14	−0.2	$7 \cdot 10^{-6}$	11	3.122
30	−0.2	$7 \cdot 10^{-6}$	11	2.477
22	−0.4	$5 \cdot 10^{-6}$	10	3.808
22	−0.4	$5 \cdot 10^{-6}$	10	3.536
22	−0.4	$5 \cdot 10^{-6}$	10	5.102

Table 2. *ANOVA with the Data of* Table 1. $R^2 = 0.849479$.

Effects	Sum of squares	Degrees of freedom	Mean squares	F_{ratio} [a])	P_{level} [b])
A: t_{dep}	34.06	1	34.06	48.64	0.0199[c])
B: E_{dep}	0.40	1	0.40	0.57	0.5304
C: C_{Cu}	14.65	1	14.65	20.93	0.0446[c])
D: pH	43.49	1	43.49	62.12	0.0157[c])
AB	7.36	1	7.36	10.52	0.0834
AC	8.04	1	8.04	11.49	0.0771
AD	10.58	1	10.58	15.11	0.0603
BC	0.01	1	0.01	0.02	0.9097
BD	11.25	1	11.25	16.06	0.0570
CD	6.04	1	6.04	8.63	0.0990
Lack-of-fit	22.67	6	3.78	5.40	0.1645
Pure error	1.40	2	0.70		
Total (corr.)	159.96	18			

[a]) $F_{ratio} = Ms_{factor}/MS_{error}$. [b]) P_{level}, probability level. [c]) Significant factor at $\alpha = 0.05$.

Cu^{II} increased. To evaluate the influence of these factors, a 2^3 factorial design was performed in the second stage.

Second Stage: 2^3 Factorial Design. This design consisted of eight individual experiments, in addition to three replicates in the central point. The values chosen for the high (+) and low (−) levels and for the central point (0) of the experimental variables were as given in *Eqns. 7–9*.

$$t_{dep}(+) = 20\ s \qquad C_{Cu}(+) = 4 \cdot 10^{-6}\ M \qquad pH(+) = 8 \tag{7}$$

$$t_{dep}(-) = 10\ s \qquad C_{Cu}(-) = 8 \cdot 10^{-6}\ M \qquad pH(-) = 10 \tag{8}$$

$$t_{dep}(0) = 15\ s \qquad C_{Cu}(0) = 6 \cdot 10^{-6}\ M \qquad pH(0) = 9 \tag{9}$$

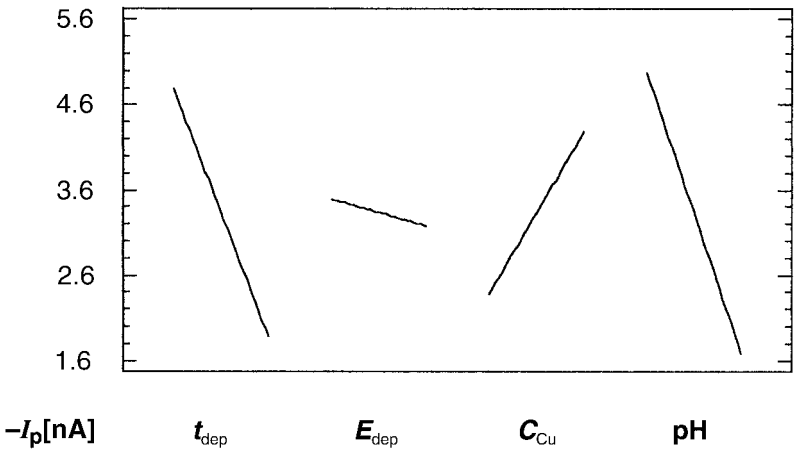

Fig. 1. *Influence of the main factors in the response variable*

Table 3. *Results of the 2^3 Factorial Design for the Optimization of Experimental Parameters in the Formation of the Complex [Cu(RIF)] by DPAdSV.* $C_{RIF} = 4 \cdot 10^{-6}$ M.

t_{dep} [s]	C_{Cu} [M]	pH	$-I_p$ [nA]
10	$4 \cdot 10^{-6}$	8	0.100
20	$4 \cdot 10^{-6}$	8	0.100
10	$8 \cdot 10^{-6}$	8	0.415
20	$8 \cdot 10^{-6}$	8	0.100
10	$4 \cdot 10^{-6}$	10	15.770
20	$4 \cdot 10^{-6}$	10	4.016
10	$8 \cdot 10^{-6}$	10	22.180
20	$8 \cdot 10^{-6}$	10	2.238
15	$6 \cdot 10^{-6}$	9	7.106
15	$6 \cdot 10^{-6}$	9	8.205
15	$6 \cdot 10^{-6}$	9	11.130

Table 4. *ANOVA with the Data of* Table 3. $R^2 = 0.928389$.

Effects	Sum of squares	Degree of freedom	Mean squares	F_{ratio}[a])	P_{level}[b])
A: t_{dep}	128.09	1	128.09	29.61	0.0322[c])
B: C_{Cu}	3.06	1	3.06	0.71	0.4889
C: pH	236.41	1	236.41	54.65	0.0178[c])
AB	9.04	1	9.04	2.09	0.2852
AC	123.09	1	123.09	28.45	0.0334[c])
BC	2.33	1	2.33	0.54	0.5395
Lack-of-fit	30.07	2	15.04	3.48	0.2234
Pure error	8.65	2	4.33		
Total (corr.)	540.74	10			

[a]) $F_{ratio} = Ms_{factor}/MS_{error}$; [b]) P_{level}, probability level. [c]) Significant factor at $\alpha = 0.05$.

The results of this design are shown in *Table 3*. The analysis of the variance (*Table 4*) shows that the deposition time and the pH, as well as their interaction, influence the peak intensity. Since neither the Cu^{II} concentration nor its interactions are significant at a confidence level of 95%, this value was set at $6 \cdot 10^{-6}$ M.

Fig. 2 presents the analysis of the principle factors of this design. Although the deposition time must be even lower to reach its optimum value, the pH must be increased. According to this criterion, a 2^2 central composite design was performed.

Third Stage: 2^2 Central Composite Design. The values corresponding to the high (+) and low (−) levels and to the central point (0) for each experimental variable were as given in *Eqns. 10–12*.

$$t_{dep}(+) = 20 \text{ s} \qquad \text{pH}(+) = 8.5 \tag{10}$$

$$t_{dep}(-) = 8 \text{ s} \qquad \text{pH}(-) = 10.5 \tag{11}$$

$$t_{dep}(0) = 14 \text{ s} \qquad \text{pH}(0) = 9.5 \tag{12}$$

The results are shown in *Table 5*. The analysis of variance (*Table 6*) reflects that only the quadratic interaction of the pH is significant at a 95% confidence interval, such that the model proposed is adequate for modelling the data, since there is no lack of fit.

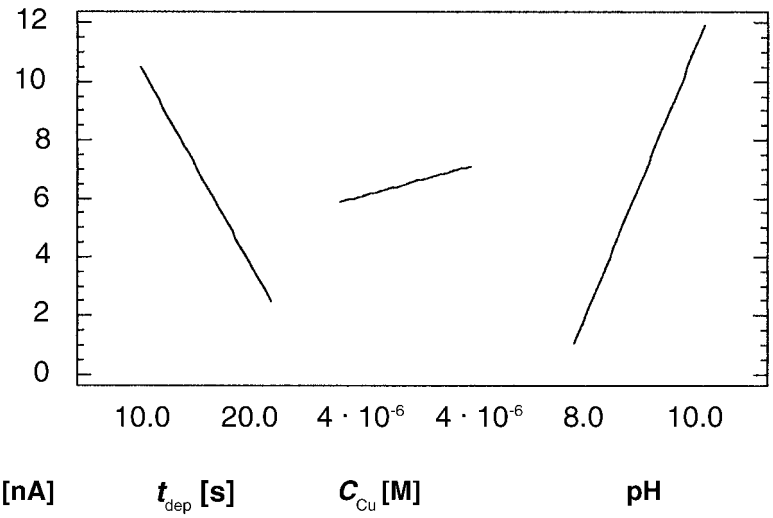

Fig. 2. *Analysis of the main factors in the factorial design 2^3, performed with the data of* Table 3

Table 5. *Results of the 2^2 Central Composite Design for the Optimization of Experimental Parameters in the Formation of the Complex [Cu(RIF)] by DPAdSV.* $C_{RIF} = 4 \cdot 10^{-6}$ M.

t_{dep} [s]	pH	$-I_p$ [nA]
8	8.50	0.100
20	8.50	6.022
8	10.50	6.764
20	10.50	1.871
5	9.50	13.920
22	9.50	9.997
14	8.09	0.100
14	10.91	3.177
14	9.50	16.370
14	9.50	22.370
14	9.50	21.030

Table 6. *ANOVA with the Data of* Table 5. $R^2 = 0.975637$.

Effects	Sum of squares	Degrees of freedom	Mean squares	F_{ratio} [a])	P_{level} [b])
A: t_{dep}	2.55	1	2.55	0.26	0.6624
B: pH	5.89	1	5.89	0.59	0.5215
AA	127.93	1	127.93	12.90	0.0695
AB	29.24	1	29.24	2.95	0.2281
BB	555.68	1	555.68	56.02	0.0174[c])
Lack-of-fit	25.16	3	8.39	0.85	0.5819
Pure error	19.84	2	9.92		
Total (corr.)	659.34	10			

[a]) $F_{ratio} = MS_{factor}/MS_{error}$. [b]) P_{level}, probability level. [c]) Significant factor at $\alpha = 0.05$.

From the level curves shown in *Fig. 3*, one can see a clearly defined maximum for pH 9.5 and a deposition time of 14 s. From this optimization process, the optimum values of *Eqn. 13* for the experimental variables in the formation of the [Cu(RIF)] complex were taken.

$$E_{dep} = -0.4\ \text{V},\ C_{Cu} = 6 \cdot 10^{-6}\ \text{M},\ \text{pH} = 9.5,\ t_{dep} = 14\ \text{s} \tag{13}$$

Fig. 4 shows the voltamograms recorded under these conditions for RIF, Cu^{II}, and [Cu(RIF)], and under which the peak current was improved upon *ca.* 25 times, giving easily quantifiable signals.

3.2. *Calibration and Detection Limit.* A calibration was carried out by least-median-squares regression (LMS) to detect the existence of anomalous points [19], which would lead to incorrect adjustments altering the sensitivity and the detection limit. The criterion is to minimize the median of squares of the differences between the experimental and the calculated values. LMS Regression has the advantage of being able to detect anomalous points whether they are 'outlier' or 'leverage', looking for a linear range when at least 50% of the data are aligned.

The strategy followed consisted of two steps. In the first, the LMS regression was used to detect anomalous points, taking a point as 'outlier' if the absolute value of the standardized residual was greater than 2.5 and as a 'leverage' if the absolute value of its resistant diagnostic was greater than 2.5. When both of these parameters were above 2.5, the point was considered as 'outlier-leverage'. In the second step, the anomalous points detected were eliminated and a regression based on the ordinary least-squares (OLS) criterion was carried out, to obtain optimal precision and accuracy of both slope and intercept.

The calibration equation for standard solutions containing between 1.992 and 2.785 μM is given by *Eqn. 14*.

$$I = -70.854 + 36.426\ C\ (R^2 = 0.9998 \text{ and } S_{yx} = 0.04265) \tag{14}$$

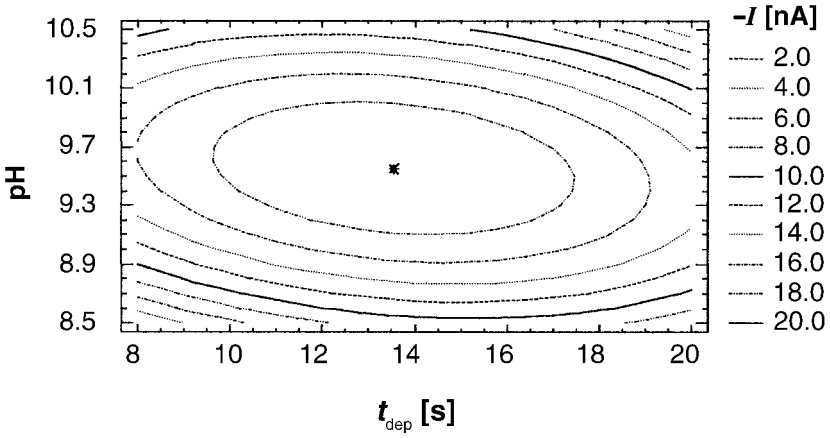

Fig. 3. *Level curves for the response variable, obtained with the data of* Table 5

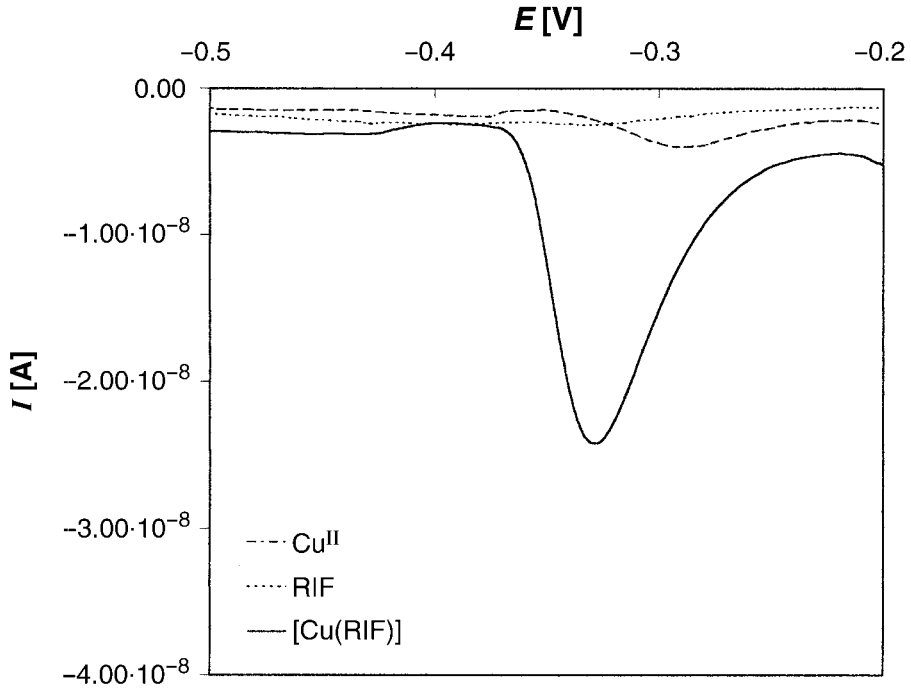

Fig. 4. *Voltammograms, obtained under the optimum conditions, for RIF* ($4 \cdot 10^{-6}$ M), *Cu^{II}* ($6 \cdot 10^{-6}$ M), *and [Cu(RIF)]*

An important characteristic of an analytical method is the detection limit, the smallest concentration of the analyte that can be detected with a specified degree of certainty. The detection limit, based on the variability of ten samples with a very low analyte concentration, was evaluated according to [21] and ISO 11843-2 [22]. At a 5% probability level chosen ($\alpha = \beta = 0.05$), the detection limit was 0.170 μM.

3.3. *Analytical Applications.* 3.3.1. *Determination of RIF in Pharmaceutical Preparations.* The determination of RIF in commercial capsules, *Rimactán*® 300 mg (*Ciba-Geigy* SDAD. ANMA.), was carried out by electrochemical techniques [9] for which it was necessary to employ the multivariate calibration methodology. This work describes a new procedure for this analysis by univariate techniques, which reduces the complexity of the analysis. To do this, a capsule was dissolved in H_2O (100 ml), and 30% HCl solution (100 μl; *Suprapur*; *Merck*, Darmstadt, Germany) was added to facilitate its complete dissolution. The concentration of RIF found, 247.37 mg ± 60.75, by standard addition of identical volumes (5 μl) of a solution of RIF ($3 \cdot 10^{-4}$ M) to a sample of the drug agrees with that given by the manufacturer.

3.3.2. *Determination of RIF in Urine Samples.* Urine samples were obtained from fasting and healthy subjects in the morning and diluted in H_2O. The analysis of RIF was made by adding the suitable quantity of the drug to the urine sample until the required concentration was obtained. The concentration of RIF in the presence of Cu^{II} was determined by the DPAdSV method with univariate techniques. The standard

additions did not give satisfactory results, due to the complexity of the sample. To analyze this type of sample, as well as those where there are overlapping signals, there are different mathematical strategies. Soft calibration methods, such as partial least squares (PLS), have proved to be a very efficient tool for the resolution of this type of samples with electrochemical techniques [13][14][24].

The calibration set consisted of nine synthetic samples containing RIF between $1.992 \cdot 10^{-6}$ and $2.627 \cdot 10^{-6}$ M. All the voltammograms were digitalized accounting for the intensity read at 135 potentials between 0 V and -0.8 V.

The PLS regression method [23][24] is widely used. It is already known that this calibration is achieved by constructing latent variables, which are linear combinations of the original variables. To maintain the maximum prediction ability of the model, it is appropriate to optimize the sum of squares in prediction (PRESS) of the PLS models, constructed with the calibration data [13][14] according to *Eqn. 15*, in which c_i is the concentration corresponding to the ith calibration sample (ith element of the vector $\boldsymbol{c}$) and $\hat{c}_{k/i}$ is the concentration estimated by the PLS model with k latent variables computed when the ith sample is removed. In practice, a more-stable estimation is obtained if, instead of eliminating only one sample to calculate the concentration of k latent variables, the highest possible fraction of the samples is cancelled. It is essential that, in the calculation process for the PLS model, neither the cancellation group nor an initial autoscaling that affects all the samples intervene in any way (full cross-validation procedure, PLSC).

$$\mathrm{PRESS}(k) = \sum_{i=1}^{m} (c_i - c_{k/i})^2 \tag{15}$$

The calculation of PRESS was done with five cancellation groups, which is to say that a PLSC model was constructed five times for a number of latent variables, eliminating 2, 2, 2, 2, and 1, respectively, of the nine polarograms [13][14].

Table 7 shows the results in percentages of explained variance and cross-validate variance (C.V.) as a function of the number of latent variables. It is obvious that, upon including new latent variables, the explained variance rises. However, if the model includes an ith latent variable not related to the response, the C.V. will not continue increasing but will rather decrease. The minimum PRESS is reached for the number of latent variables that give the maximum C.V. According to this criterion, one must take four latent variables.

The concentration found with this model for RIF was compared with the true value. The average relative absolute error obtained was 2.31%.

Table 7. *Variance Explained in the Blocks of Predictors* (X) *and Response* (Y) *and Cross-Validate Variance* (C.V.) *for the Concentration of RIF by the PLS Model Constructed*

Latent-variables index	Explained variance of Y block [%]	C.V. Explained variance of Y block [%]	Variance of X block [%]
1	20.140	81.782	87.196
2	93.293	92.368	94.589
3	99.694	99.374	97.769
4	99.877	99.615	98.752
5	99.921	99.551	98.984

This PLSC-calibration model was applied to a test set of four urine samples with a known concentration of RIF ($2 \cdot 10^{-6}$ M). The concentration found was $2.397 \pm 0.486 \cdot 10^{-6}$ M ($n = 4$ and $\alpha = 0.05$).

3.4. *Influence of Different Metals.* Analysis of the possible complexes formed by RIF and different metals was studied, under the optimized conditions described above for Cu^{II}. In this way, Pb^{II}, Cr^{III}, Cr^{VI}, Fe^{II}, Fe^{III}, Cd^{II}, and Zn^{II} were tested without successful results, which does not imply that, under other conditions of pH, t_{dep}, E_{dep}, and C_{metal}, the complex may be formed.

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