ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 1015–1028

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Development and validation of a differential pulse polarographic method for quinolinic acid determination in human plasma and urine after solid-phase extraction: a chemometric approach

Sandra Furlanetto [a,*], Sergio Pinzauti [a], Enzo La Porta [a], Alberto Chiarugi [b], Paola Mura [a], Serena Orlandini [a]

[a] *Department of Pharmaceutical Sciences, University of Florence, Via G. Capponi 9, 50121 Firenze, Italy*
[b] *Department of Preclinical and Clinical Pharmacology, University of Florence, V. le Pieraccini 6, 50134 Firenze, Italy*

Received 11 November 1997

Abstract

A chemometric approach was applied for determining quinolinic acid in human plasma by differential pulse polarography after solid phase extraction. A fractional factorial design was used to examine the significant experimental variables for the peak height maximization. A Doehlert design, which allowed a sequential response surface methodology to be performed, was applied to the variables scan rate and drop size. The results indicated that the scan rate had the greatest effect on the response peak height. The linear range was extended from 8.52×10^{-8} to 1.34×10^{-5} M and the limit of detection was 2.9×10^{-8} M. The validation process consisted of a pre-validaton study followed by the main validation in the plasma matrix. The robustness and the intermediate precision were evaluated by means of experimental design. A $3^4//9$ screening symmetric matrix and a central composite design were used to optimize the solid phase extraction procedure of the analyte from human plasma using anion exchange cartridges. The goal was to select the best retention, wash and elution solvents and their volumes in order to maximize the extraction efficiency using as the response the polarographic peak height. An extraction efficiency of 90% was found. The method was also applied to the determination of quinolinic acid in urine and the mean concentration in human plasma and urine, was found to be 3.7×10^{-7} and 4.9×10^{-5} M respectively.

Keywords: Quinolinic acid; Determination; Optimization; Validation; Robustness testing; Differential pulse polarography; Experimental design; Biological fluids; Endogenous metabolite; Solid-phase extraction; Sample preparation

1. Introduction

Quinolinic acid (QUIN; Fig. 1), an excitotoxic metabolite formed in vivo from tryptophan, acts

* Corresponding author. Fax: +39 55 240776; e-mail: smr@farmfi.scifarm.unifi.it

0731-7085/98/$19.00
PII S0731-7085(98)00067-3

as a competitive agonist of the *N*-methyl-D-aspartate receptors. What has emerged recently is the evidence that concentrations of quinolate can increase to levels that would be toxic to central neurones, especially in situations of infection and inflammation [1].

QUIN concentration is substantially higher in plasma than in the brain, but an increase in plasma levels leads to enhanced QUIN concentrations in brain tissue probably due to an increase in blood brain barrier transport [2]. QUIN is excreted in urine and the amount of urinary QUIN is one of the criteria to evaluate vitamin B6 deficiency [3].

In agreement with these observations, the determination of QUIN in biological fluids is important and can be useful for elucidating biochemical mechanisms.

Determination of QUIN in biological fluids has been carried out by gas chromatography [4], liquid chromatography [5,6], gas chromatography-mass spectrometry [7,8], high-voltage electrophoresis [9] and by capillary zone electrophoresis [10].

QUIN is an electroactive molecule that undergoes irreversible electrochemical reduction on a dropping mercury electrode [11–13]. However, no application of the polarographic method to the analysis of real samples, such as plasma or urine, was reported.

QUIN plasma levels were found to be about 7×10^{-7} M [1,2], and QUIN in daily urine was found to be about 35 μmol [5]. An adsorptive stripping voltammetric method was developed in our laboratory for assaying kynurenic acid, another tryptophan metabolite, and the optimized method allowed nM concentrations to be determined [14]. Although QUIN is electroactive, it does not exhibit surface properties and, in preliminary experiments, appeared unable to adsorb onto the hanging mercury drop electrode. Therefore, a differential-pulse polarographic method was developed for its determination in human plasma and urine, and validated for the plasma matrix. An experimental design strategy was applied for the optimization and validation. Experiments to be carried out were previously planned, according to experimental design, within the factor space in order to obtain maximum information from a series of carefully selected experiments. The validation of the method consisted of a pre-validation phase followed by a main validation phase in the matrix of interest. During the pre-validation process, experimental design was useful for testing the performance of the analytical parameters for which simultaneous variation of variables was required. Thus, intermediate precision and robustness were assessed by means of statistical experimental design [15].

A chemometric optimization may also be useful in sample preparation procedures for the selection of optimum solid-phase extraction (SPE) parameters and little information has been reported on the systematic optimization of the sample preparation [16,17]. Several factors influence the sample cleanup by SPE and an attempt to evaluate their influence on the extraction efficiency by means of experimental design is presented in this paper. The goal was to maximize the extraction efficiency of QUIN from human plasma using anion-exchange cartridges.

2. Experimental

2.1. Materials

All chemicals and reagents used were of analytical-reagent grade with no further purification. Quinolinic, picolinic, nicotinic and kynurenic acids were purchased from Sigma (St. Louis, MO) and stored at −18°C. Britton–Robinson universal buffer (B-R) (pH 1.8) (0.04 M boric, phosphoric and acetic acids) was used as the supporting electrolyte and 0.5 M B-R universal buffer (pH 1.6) was used as the elution solvent. Clarke and Lubs (C-L) buffer (pH 1.1) (25 ml 0.2 M potas-

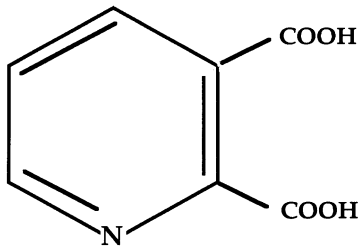

Fig. 1. Chemical structure of quinolinic acid.

sium chloride and 50 ml 0.2 M hydrochloric acid made up to a volume of 100 ml with water) was used in the experimental design. Ultrapure reagent-grade water was obtained with a Milli-Q system (Millipore/Waters, Milford, MA) and was used to prepare all solutions. SPE were performed on Bond Elut SAX cartridges (Varian), a strong anion-exchange resin (500 mg, sorbent bed; 3 ml, reservoir).

2.2. Biological samples

Plasma samples were obtained from young healthy volunteers (Banca Militare del Sangue, Florence, Italy) and stored frozen until measurement. For experimental design a pool of plasma samples was used. Human urine was collected and stored at 4°C until analyzed within 6 h.

Phosphate buffered saline was used as the reconstituted plasma sample. The composition was as follows: 20 mg potassium chloride, 800 mg sodium chloride, 20 mg monopotassium phosphate and 115 mg disodium phosphate, were dissolved in 100 ml water. QUIN standard solution (100 μl) was added and, in order to mimic the drug–protein interactions, 4 g bovine serum albumin (Sigma) were also added.

2.3. Equipment

The polarographic experiments were carried out with an AMEL 433 polarographic analyzer (Amel, Milan, Italy) incorporating a magnetic stirrer and a three-electrode system consisting of a hanging mercury drop working electrode coupled with a Ag/AgCl reference electrode and a platinum wire as auxiliary electrode. The AMEL analyzer was connected to a personal computer and the data handling, storage, printout and graphics were obtained by means of special AMEL software. For the intermediate precision study, two polarographic AMEL 433 were used. pH measurements were obtained with a Metrohm 691 pH meter (Metrohm, Herisau, Switzerland). The experimental design was generated, and statistical analysis of the data was performed, using Nemrod 3.1 software (LPRAI, Université de Marseille III, France).

The GC-MS system consisted of a HP6890 GC-HP5973 MS (Hewelett-Packard) equipped with a HP6890 (Hewelett-Packard) automatic injector. A HP 5 MS 30 m × 0.25 mm × 0.25 μm chromatographic column (Hewelett-Packard) was used. An ALF Micro Centrifugette 4214 and Ultra Speed-Vaac concentrator (Savant) were used for sample preparation before GC-MS analysis.

2.4. Polarographic analysis

For preparing the standard solution, an accurately weighed amount of QUIN (20 mg) was dissolved with 10 ml 0.01 M sodium hydroxide. The resulting solution was diluted to 100 ml with water and it was stable for at least four days at room temperature. Working solutions were prepared daily by diluting 0.50 ml standard solution to 10 ml with water.

Working solutions of picolinic, nicotinic and kynurenic acids were prepared in water at a concentration level of about 1.5 μg ml^{-1}.

2.4.1. Procedure

The polarographic procedure was carried out as follows: 10 ml 0.04 M B-R buffer (pH 1.8) was transferred into the polarographic cell and deaerated by bubbling nitrogen-free oxygen for 10 min in the first cycle and 30 s for each successive cycle. A differential pulse scan, with a pulse amplitude of 60 mV, scan rate of 1 mV s^{-1} and pulse duration of 20 ms, towards more negative potentials, was performed from -0.65 to -0.90 V in order to obtain the polarogram. The drop size was 65 a.u. The polarographic cycle was repeated twice and the mean was obtained.

Before adding a sample aliquot to the supporting electrolyte, a polarogram of the blank was recorded under the same conditions. Determination of QUIN was accomplished by the method of standard additions (three additions). The peak height was used to quantify QUIN.

2.4.2. SPE

The SAX cartridge was activated with 5 ml methanol and conditioned with 10 ml 0.01 M hydrochloric acid. The sample (5 ml human plasma, or 5 ml reconstituted plasma, adjusted to

pH 9 with 0.1 M sodium hydroxide) was then loaded. Water (3 ml) was passed through the column and washing was then carried out with 2 ml monopotassium phosphate. QUIN was eluted with 4 ml 0.5 M B-R buffer (pH 1.6).

2.4.3. Analysis in plasma

The eluate from SPE was made up to 10 ml volume with water (plasma solution), transferred into the cell and directly analyzed by dp polarography.

The determination of QUIN in plasma was carried out by means of the standard additions method (three additions) and each addition consisted of 100 μl QUIN working solution.

To assess the accuracy and repeatability, a plasma solution polarogram was recorded and four 100 μl additions of QUIN working solution were made. The plasma solution polarogram was considered as a blank and subtracted from the others in order to subtract the current due to the QUIN endogenous quantity. The first addition was considered the unknown.

For the linearity study the calibration plot was obtained using as a blank the plasma solution. Seventeen additions of QUIN working solution were then made (the first two additions of 15, the third of 50, the fourth of 100 and successive additions of 250 μl). The blank plasma solution polarogram was subtracted from the others.

2.5. GC-MS analysis

Trichloroacetic acid (100 μl) and 100 μl 1 μM [^{13}C]quinolinic acid (internal standard) were added to an equal volume of plasma. After centrifugation for 10 min at 14 000 rpm, the supernatant was collected in a 1 ml glass vial and lyophilized in a speed-vac concentrator. Then 100 μl trifluoroacetyl–imidazole and 100 μl hexafluoro-2-propanol were added to the lyophilized samples that were then derivatized at 80°C for 1 h. The samples were then washed with 100 μl water and extracted with 100 μl heptane, they were frozen at −80°C and the heptane fraction was collected; 2 μl of this was injected into the GC-MS system. The carrier gas was helium at the constant flow of 1.2 ml min^{-1}. The GC oven temperature program in a pulsed splitless mode was 1 min at 80°C, then ramped to 135°C at 10°C min^{-1}. The temperature was then increased to 300°C at 25°C min^{-1} and it was maintained for 6 min. The injector and transfer line temperatures were 230 and 270°C, respectively. The MS detector operated in electron impact at 70 eV; selected ion monitoring mode was used on six ions with a dwell time of 50 ms for each ion. The selected ions were 272.1/300.1/448.1 for quinolinic acid and 278.1/307.1/455.1 for [^{13}C]quinolinic acid. For quantitation, the ratio between the peak-area of the 272 and 278 ions was selected and related to a previously constructed calibration curve.

3. Results and discussion

Preliminary experiments, carried out with different supporting electrolytes, showed that QUIN did not exhibit surface properties. A voltammogram of a 1×10^{-6} M QUIN solution with a hanging drop mercury electrode was recorded and compared with a voltammogram obtained under the same conditions but after an 80 s accumulation time at 0 V potential under stirring conditions. No difference in the peak current was observed, thus indicating that it was impossible to apply the adsorptive stripping voltammetric technique to QUIN determination.

Therefore, a differential-pulse polarographic method was developed and optimized for QUIN determination. Some general information about the polarographic reduction of QUIN has been described [11–13] but no application of the method to biological sample analysis was reported.

A preliminary study pointed out that pH 1.8 B-R buffer and pH 1.1 C-L buffer seemed to be the best supporting electrolytes and both were studied with a two level statistical experimental design. Differential-pulse was selected as the scan mode, thus the pulse duration and the pulse amplitude were to be optimized. The dropping time was kept at 1 s, whilst the other parameters chosen for optimization were the drop size and the scan rate.

The optimization process involved the use of an experimental design with the aim of constructing the experiments so that an analysis of results yielded the maximum amount of information with minimum effort and cost.

3.1. Method set-up by experimental design

The optimization strategy involved a two-level fractional factorial design followed by a Doehlert design that allowed a sequential response surface methodology to be performed.

In the first phase the main effects of the five considered factors (drop size, x_1; pulse duration, x_2; pulse amplitude, x_3; scan rate, x_4; and type of supporting electrolyte, x_5) and all the first-order interactions were studied, keeping the total number of trials within reason. The considered response was peak height and the model studied (Eq. (1)) was:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_5 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{14}x_1x_4 + b_{15}x_1x_5 + b_{23}x_2x_3 + b_{24}x_2x_4 + b_{25}x_2x_5 + b_{34}x_3x_4 + b_{35}x_3x_5 + b_{45}x_4x_5 \quad (1)$$

Since fractional factorial design measures the main effects and some interactions with a number of trials of 2^{k-p}, where p is an arbitrary number less than k [18], a resolution IV fractional factorial design 2^{5-1}, with generator 12345, was employed to estimate the model parameters. The matrix also included four experiments at the central level of each factor in order to test the model linearity and to obtain an estimate of the experimental error. From the experimental point of view, the five parameters examined had to be independently adjusted to their central (0), high (+1) and low (−1) extreme value levels in 20 different combinations, as determined within the proposed fractional factorial design. The selection of the experimental domain for the factors is critical for generation of useful data and maximum (+1) and minimum values (−1) for each factor were fixed on the basis of our experience (Table 1). The 20 experiments were carried out with a QUIN in cell concentration of 6.1×10^{-7} M. Randomization was used to obtain a random distribution of small, unknown systematic errors, thus allowing treatment of the experimental error as if it had only one component: random error [18].

Table 1
Factors and their experimental domain boundaries

Factor	Low level (−1)	High level (+1)
Drop size (a.u.)	20	60
Pulse duration (ms)	20	40
Pulse amplitude (mV)	30	60
Scan rate (mV s^{-1})	10	30
Type of supporting electrolyte	C-L	B-R

The assumed regression model was found to be significant and the statistical analysis of the coefficients [19] showed that all main effects ($b_1 - b_5$) and the interactions drop size–scan rate (b_{14}), pulse duration–pulse amplitude (b_{23}), pulse duration–scan rate (b_{24}), pulse amplitude–type of supporting electrolyte (b_{35}) and pulse amplitude–scan rate (b_{34}), were significant (Table 2).

In particular, the best supporting electrolyte was found to be B-R (pH 1.8) buffer and the variables pulse duration and pulse amplitude were fixed at their lower (20 ms) and higher (60 mV) values, respectively, according to the principal and interaction effects.

The validity of the model was checked by running four trials at the center of the experimental domain. Since the mean of the determined values at this level was not in good agreement with the

Table 2
Estimated coefficients of Eq. (1) in the text

Coefficient	Estimate	Coefficient	Estimate
b_0	59.00	b_{14}^a	−6.75
b_1^a	9.75	b_{15}	−0.88
b_2^a	−9.13	b_{23}^a	−5.88
b_3^a	20.50	b_{24}^a	3.63
b_4^a	−18.50	b_{25}	−1.25
b_5^a	3.63	b_{34}^a	−3.25
b_{12}	−0.88	b_{35}^a	5.88
b_{13}	1.50	b_{45}	−0.38

[a] Significant coefficient.

Table 3
Matrix of the first Doehlert design

Experiment no.[a]	x_1	x_2
5	1.0	0
3	−1.0	0
10	0.5	0.866
6	−0.5	−0.866
1	0.5	−0.866
9	−0.5	0.866
7	0	0
8	0	0
2	0	0
4	0	0

[a] Randomized order.

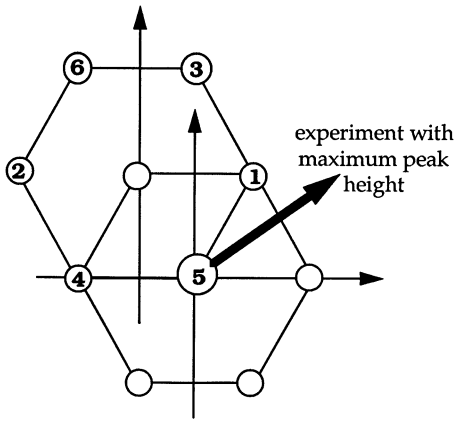

Fig. 2. Sequence of Doehlert designs toward the optimum.

value given by the model, the linear model could not be considered valid.

To obtain a second-degree model, describing the response as a quadratic function of the two remaining variables, a Doehlert design was applied.

The Doehlert design structure allowed sequential response surface methodology to be performed. By building sequential designs, it was possible to move the Doehlert toward the region in which the optimum values of the variables studied were simultaneously obtained. The Doehlert design requires $k^2 + k + n$ experiments, where k is the number of variables and n is the number of center points. For two variables the Doehlert is a hexagon and each experiment corresponds to a vertex of such a geometrical structure according to the experimental matrix reported in Table 3. In this way one factor is studied at three levels and the other at five levels.

In this study the factors scan rate and drop size were studied at three and five levels, respectively. The experimental domain was 5–13 mV s^{-1} for scan rate and 40–60 a.u. for drop size. The shape of the Doehlert permits great mobility across the experimental domain of the variables. Therefore, after a first Doehlert design, a new Doehlert design was centered onto the experiment that had given the maximum peak height in the first design (Fig. 2). The second Doehlert design contained four points of the first (share of a square face) and three new points. The block effect, i.e. the variation of the response due to different time of analysis, was evaluated analyzing a regression model containing the two considered factors and also a Z factor corresponding to the block variable. The matrix considered was a matrix that contained all the points of the first and the second Doehlert design and the responses processed were that obtained in each point. Since the coefficient of the Z factor was not significant, the absence of the block effect was assessed and only the experiments corresponding to the three new points were performed in a new experimental domain of 1–9 mV s^{-1} for scan rate and 45–65 a.u. for drop size. The corresponding matrix is reported in Table 4. Replicates with the 0 codified values of the variables were performed in order to validate

Table 4
Matrix of the second Doehlert design

Experiment no.[a]	x_1	x_2
[b]	−0.5	−0.866
[b]	1.0	0
[b]	0.5	−0.866
[b]	0	0
1	1.0	−1.732
3	1.5	−0.866
4	0	−1.732
5	0	0
2	0	0
6	0	0

[a] Randomized order.
[b] Points of the first Doehlert design.

Table 5
Experimental plan of the second Doehlert design and the responses obtained

Experiment no.[a]	Drop size (a.u.)	Scan rate (mV s^{-1})	Response (nA)
[b]	45	5	147
[b]	60	9	141
[b]	55	5	172
[c]	50	9	124
1	60	1	205
3	65	5	196
4	50	1	184
5	50	9	138
2	50	9	138
6	50	9	135

[a] Randomized order.
[b] Points of the first Doehlert design.
[c] Mean of responses at the center of the first Doehlert design.

the model by means of an estimate of the experimental variance. The experimental plan, together with the responses obtained, is reported in Table 5.

The regression model was found to be valid and significant. From the response surface (Fig. 3) it was possible to select the optimized conditions. The maximum response was obtained for a scan rate at its lowest level (1 mV s^{-1}) and a drop size at its higher level (65 a.u.).

The predictive capacity of the model was validated according to a previously reported procedure [20]. Using the optimized conditions: B-R buffer (pH 1.8); pulse amplitude, 60 mV; pulse duration, 20 ms; scan rate, 1 mV s^{-1} and drop size, 65 a.u.; QUIN showed a reduction peak at − 700 mV versus an Ag/AgCl reference electrode.

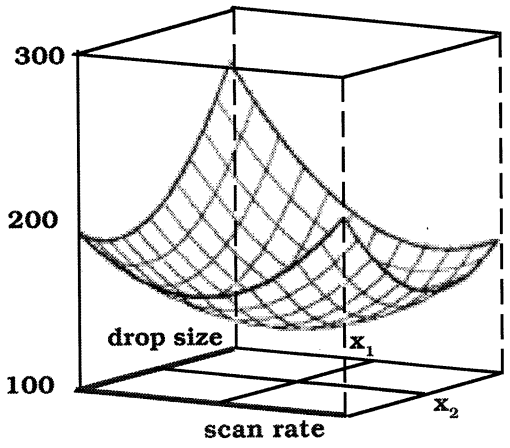

Fig. 3. Response surface for the scan rate (x_2) against the drop size (x_1).

3.2. Pre-validation step

The method developed was pre-validated using a QUIN working solution according to the guidelines of ICH3 [15] for the validation of analytical methods. The performance analytical parameters examined were: robustness, selectivity, linearity, range, limit of quantitation (LOQ), limit of detection (LOD) and accuracy and precision as a measure of repeatability and intermediate precision.

3.2.1. Robustness testing

Robustness testing was performed in order to obtain information about those critical parameters affecting the response (polarographic peak height) [14,15]. The robustness of a method can be tested using an experimental design in order to study the simultaneous variation of the factors. As a result of the data analysis, one is able to indicate which of the tested factors are not robust for the considered response. When factors that are not robust are detected one can decide to change the method or to control the factor in question more strictly.

Robustness testing involves the selection of the factors and of the experimental domain in which to study them. The effect on the response of small

Table 6
Experimental domain of the factors studied in the robustness testing

Factor	Low level (−1)	Central level (0)	High level (+1)
Pulse duration (ms)	19	20	21
Pulse amplitude (mV)	58	60	62
Drop size (a.u.)	62	65	68
Supporting electrolyte concentration (M)	0.0064	0.04	0.25

variation with respect to the optimized values is evaluated. In this work the four factors considered, studied at three levels, were: pulse duration (x_1); pulse amplitude (x_2); drop size (x_3) and concentration of the supporting electrolyte (x_4). The parameter, scan rate, was not considered due to instrumental settings that did not allow small variation of the value. The experimental domain explored for each factor is reported in Table 6 and the center corresponded to the optimized condition.

Although robustness testing involves deliberate small variations in the method parameters, for the factor concentration of the supporting electrolyte, a large experimental domain was studied in order to evaluate the effect of relevant change, as those can occur due to the sample preparation procedure.

A quadratic model with first order interactions, was postulated and a D-optimal design [14,21,22] was used to select, from a central composite design for four variables with a cubic experimental domain, a subset of experiments in order to estimate with accuracy the model coefficients with a minimum number of experiments. A 23-run matrix, including four replicates at the center of the experimental domain, was found to be the best compromise between the number of trials and information quality. This approach allowed the factors to be studied at three levels and a response surface study to be performed with a minimum number of experiments. The experiments were carried out according to the chosen design (Table 7) and the model assumed was found valid and significant. Only the factor concentration of the supporting electrolyte (x_4) was significant for the regression model assumed. The response surface for the supporting electrolyte against the pulse amplitude, maintaining the other factors to their optimized values (Fig. 4), shows that only near the center, that is near the optimized conditions, the method is robust, thus a precautionary statement should be included in the procedure for this factor.

3.2.2. Selectivity

Due to the shift of the reduction potential, of kynurenic, nicotinic and picolinic acids, toward more negative values (about −840 mV), selectivity against these potentially interfering metabolites was assessed.

3.2.3. Linearity, range and limits

Applying the optimized conditions, the calibration curve gave a straight line in a 25-level concentration range from 8.52×10^{-8} to 1.34×10^{-5} M with a correlation coefficient R^2 of 0.9995 and an R^2_{CV} [22] of 0.9993. The calibration curve equation was $y = 0.039$ (nA $\times 10^7$ M)$x - 0.052$ (nA).

The RSD of the regression line, *sd*, was calculated [23]. The slope of the regression line and *sd* were the basic parameters to calculate the LOD and LOQ. In particular the LOD, equal to 3 *sd* slope^{-1}, was found to be 2.9×10^{-8} M and was validated by means independent measurements carried out with QUIN at a concentration level near LOD. The LOQ, defined as 10 *sd* slope^{-1}, was found to be 9.7×10^{-8} M, that is near the experimental LOQ (8.52×10^{-8} M), corresponding to the lowest concentration level of the calibration curve.

3.2.4. Accuracy

Since QUIN is an endogenous metabolite, its quantitation in biological fluids and tissues re-

Table 7
D-optimal design matrix and the responses obtained

Experiment no.[a]	x_1	x_2	x_3	x_4	Response (nA)
3	−1	−1	−1	−1	146
8	1	−1	−1	−1	145
15	−1	1	−1	−1	165
21	1	1	−1	−1	149
9	−1	−1	1	−1	172
22	1	−1	1	−1	156
1	−1	1	1	−1	153
13	−1	−1	−1	1	150
14	1	−1	−1	1	130
18	−1	1	−1	1	147
5	1	1	−1	1	139
10	−1	−1	1	1	144
11	1	−1	1	1	139
6	−1	1	1	1	145
23	1	1	1	1	141
12	1	0	0	0	174
20	0	1	0	0	187
4	0	0	1	0	167
17	0	0	0	−1	161
19	0	0	0	0	168
16	0	0	0	0	176
2	0	0	0	0	167
7	0	0	0	0	159

[a] Randomized order.

quired the standard additions method. Accuracy was evaluated by means of the standard additions method (three additions) and measured by the bias (that is the difference of the mean value from the true or accepted reference value) [24] at three levels: 8.52×10^{-8}, 5.78×10^{-7} and 1.14×10^{-6} M; the bias was found to be 3.71, 3.45 and 5.75%, respectively.

3.2.5. Precision

The precision, as a measure of repeatability, was evaluated at the three different concentration levels above using five replicates. The RSD was 3.47, 4.58 and 5.01%, respectively.

Since it is not always relevant or practical to measure reproducibility, the intermediate precision study was performed [15,24].

The factors considered were analyst, equipment and time; the considered response was the determined QUIN concentration obtained using the standard additions method. A liner model ($y = b_0 + b_1x_1 + b_2x_2 + b_3x_3$) was assumed and a 2^3 full factorial design was employed to estimate the model coefficients. Each experiment was repeated three times in order to evaluate the experimental variance and the analyses were carried out with QUIN concentration in the cell of 2.63×10^{-7} M. The regression model was found to be valid but not significant, thus indicating that no factor considered influenced the response (RSD = 7.1%, $n = 24$).

3.3. Analysis in plasma

3.3.1. SPE optimization

The determination of QUIN in plasma by the developed differential pulse polarographic method, required an SPE step in order to eliminate the interference compounds and to concentrate the analyte.

QUIN is a pyridinedicarboxilic acid and it is soluble in alkalies and almost insoluble in organic solvents, thus, anion exchange cartridges were used for the clean-up procedure and the sample

was adjusted to pH 9 in order to obtain the analyte in the anionic form.

An experimental design approach to find the optimum SPE procedure was employed and the goal was to select the best retention, wash and elution solvents and their volumes, in order to maximize the extraction efficiency using as the response the current of the polarographic signal. Each experiment involved conditioning of an extraction cartridge with methanol (5 ml), loading of the sample (5 ml) followed by water (3 ml) in order to remove salts and proteins which may be loosely bound to, or trapped in the interstices of the packing material. The other factors were changed according to the established experimental plan.

In the first phase, the response was modelled according to a Free-Wilson model for four qualitative variables studied at three levels:

$$y = A_0 + A_1A + A_2A + B_1B + B_2B + C_1C + C_2C + D_1D + D_2D$$

where A is the retention solvent, B is the first wash solvent, C is the second wash solvent and D is the elution solvent.

A screening symmetric matrix ($3^4//9$) (Table 8) which allowed the four factors to be studied at three different levels with nine experiments, was used to estimate the model coefficients [25]. Each level corresponded to a different solvent as reported in Table 9. The nine experiments were performed using 5 ml pooled plasma and 10 ml retention solvent (A), 2 ml of the first (B) and the second (C) wash solvent and 3 ml elution solvent (D). Each eluate was dried under nitrogen stream and reconstituted with 10 ml of B-R buffer (pH 1.8) (0.04 M) before the polarograhic analysis. The responses measured (peak height) were processed and from the graphic analysis of effects it was possible to obtain preliminary results (Fig. 5). The advantage of this plot is that the numerical values of the effects are displayed. Since there were no degrees of freedom, Lenth's approach was used to obtain an estimate of the pseudo-SD (PSD) in order to define the confidence interval [26]. In particular the effects that exceed the solid reference line are those significant for the response together with the effects that exceed the dotted line [25]. In the right panel are represented the positive effects, that is the effects relative to those variables which determine an increase in the response, while in the left panel are reported the negative effects, that is the effects relative to those variables which determine a decrease in the response. This plot showed that 0.01 M sodium hydroxide (the retention solvent), 0.01 M formic acid (the second wash solvent) and 0.5 M sodium citrate (the elution solvent), were inappropriate, leading to a response lowering, while 0.01 M hydrochloric acid, 0.01 M monopotassium phos-

Table 8
$3^4//9$ Screening symmetric matrix for SPE optimization

Experiment no.[a]	x_1	x_2	x_3	x_4
5	0	0	0	0
3	1	0	1	1
7	2	0	2	2
6	0	1	1	2
1	1	1	2	0
9	2	1	0	1
4	0	2	2	1
8	1	2	0	2
2	2	2	1	0

[a] Randomized order.

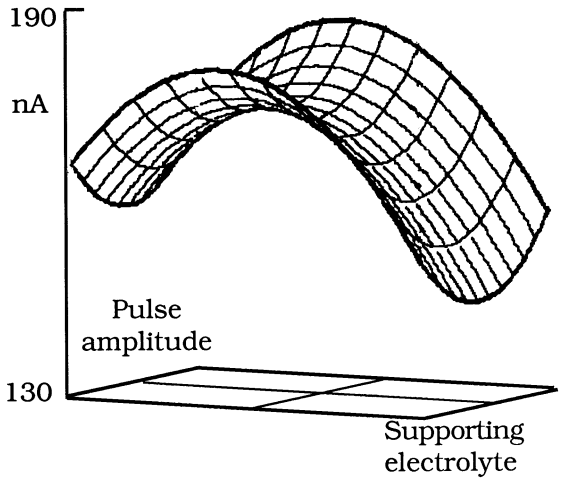

Fig. 4. Robustness testing response surface for the supporting electrolyte concentration against the pulse amplitude, maintaining the drop size at 65 a.u., the pulse duration at 20 ms and the scan rate at 1 mV s^{-1}.

Table 9
Assigned solvents for each factor studied

Factor	Level 0	Level 1	Level 2
A (retention solvent) (0.01 M)	NaOH[a]	HCl	Na_2HPO_4
B (1st wash solvent) (0.01 M)	CH_3COOH	Na_2HPO_4	$NaHCO_3$
C (2nd wash solvent) (0.01 M)	HCl	HCOOH	KH_2PO_4
D (elution solvent) (0.5 M unless otherwise indicated)	HCl	HCOOH[a]	sodium citrate

[a] Concentration was 3 M.

phate and 0.5 M hydrochloric acid seemed to be the best retention second wash and elution solvents, respectively. Moreover the first wash solvent seemed not to be important for the response.

At this point a new experimental design was performed with reconstituted plasma in order to study the improvement of the extraction efficiency with simpler sample handling. On the basis of the results obtained the following variations were made: 0.01 M borax was used instead of 0.01 M sodium hydroxide as the retention solvent and 0.5 M B-R buffer (pH 1.6) was used as the elution solvent instead of 0.5 M sodium citrate. The latter choice was due to the fact that acidic solvents seemed to be the best elution solvents and acidic B-R buffer was the supporting electrolyte in which the polarographic analysis was carried out.

The same experimental matrix $3^4//9$ reported in Table 8 with nine experiments and the same Free–Wilson model of the first screening phase were used. The treatment of the measured responses pointed out, by means of graphic analysis of effects, that the only significant factor was the

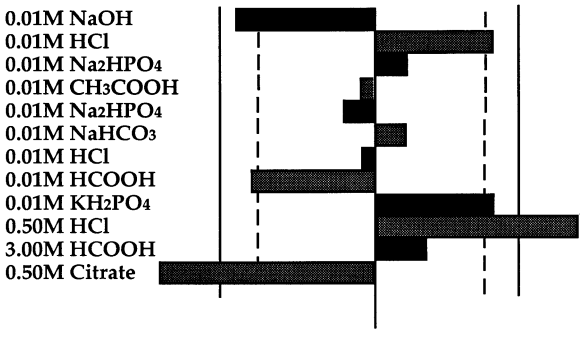

Fig. 5. Graphic analysis of the effects during SPE optimization. Right panel, positive effects; left panel, negative effects.

retention solvent, and in particular the best retention solvent was 0.01 M hydrochloric acid. As regards the other variables, these seemed not to be significant for the response. Thus, combining the information obtained from the first and the second design, it was decided to use 0.01 M hydrochloric acid as the retention solvent, to use only one wash with 0.01 M monopotassium phosphate and to use B-R buffer as the elution solvent in order to avoid the time consuming evaporation process. The choice to use only one wash was due to the fact that also in the first design, carried out with real plasma samples, this factor seemed not to be significant for the response.

In the final step of the SPE clean-up optimization, the volume of the retention (*A*), wash (*C*) and elution (*D*) solvents was optimized. A quadratic model was postulated and a central composite design, with three replicates at the center of the experimental domain, was employed to estimate the coefficients. A cubic experimental domain was explored in order to study the factors at three levels. The experimental domain of the factors considered is reported in Table 10 and reconstituted plasma was used. The analysis of the results pointed out that the regression model was valid but not significant. The response surface obtained, having fixed *C* at its lower level (2 ml) (Fig. 6), showed that, as *A* or *D* are varied over the factor space, there was only a relatively small change in the observed response. This is clearly a robust zone of the optimized method. Thus 10 ml was the optimal retention volume chosen in order to avoid consuming time and money, 2 ml was the volume for the wash solvent and 4 ml was the volume of the elution solvent in order to maximize the extraction efficiency whilst maintaining robust conditions.

Table 10
Experimental domain of the factors in SPE step volume optimization

Factor	Low level (−1)	Central level (0)	High level (+1)
A (retention solvent) (ml)	10	15	20
C (wash solvent) (ml)	2	3	4
D (elution solvent) (ml)	3	4	5

Since QUIN is an endogenous metabolite, to find the yield of the SPE procedure with real plasma samples is a very difficult task, thus reconstituted plasma was used and the yield was about 90%.

3.3.2. Validation

The difficulty in validating a bioanalytical method for an endogenous metabolite, that is always present in the matrix at unknown concentration, was reported previously [27].

The validation in plasma of the developed differential pulse polarographic method was carried out with plasma solutions after SPE. The analytical performance parameters evaluated were selectivity, just assessed in the pre-validation step, linearity and range, accuracy and precision as a degree of repeatability.

Selectivity against kynurenic, nicotinic and picolinic acids was confirmed in the plasmatic matrix where QUIN showed a reduction peak at − 735 mV. The effect of the different matrices from six individuals was evaluated in order to test the interference of unexpected endogenous compounds [27,28]. The triptophan metabolites were added to the plasma solution at a concentration level of 1×10^{-7} M and no interference was observed.

To assess the linearity a calibration plot, using as a blank a plasma solution, was constructed. The polarogram of plasma solution was subtracted from the others in order to eliminate the current due to the unknown QUIN concentration and the linearity was extended, in a 17-level concentration range, from 8.52×10^{-8} to 1.34×10^{-5} M (Fig. 7). The regression line equation was $y = 0.0318$ (nA $\times 10^7$ M)$x - 0.054$ (nA) with a R^2 of 0.9991 and a R^2_{CV} of 0.9986. This confirmed that the unexpected endogenous compounds present in the plasma solution did not interfere with the polarographic reduction of QUIN onto the drop mercury electrode. Furthermore, the large dynamic range found allowed the method to be used for quantifying QUIN in plasma where large variability could be present.

The accuracy was assessed using as a blank a plasma solution. The first addition of the standard solution was considered unknown and determined by means of the standard additions method. The polarogram of the plasma solution was subtracted from the others and the peak height was used to quantify QUIN. Three different concentration levels (five replicates) were tested: 1.0×10^{-7}, 5.5×10^{-7} and 1.2×10^{-6} M. The estimated bias was 4.21, 3.28 and 6.35%, respectively and RSD was 5.47, 4.23 and 4.59%, respectively.

The results generated by the differential pulse polarographic method were compared with those obtained by a GC-MS method. Six replicates of a pool of five plasma samples from different individuals were performed and the recovery was 113.5%, the mean concentration found with the GC-MS method being 1.63×10^{-7} M and the mean concentration found with the differential pulse polarographic method being 1.85×10^{-7} M .

3.3.3. Plasma levels

The optimized and validated method was applied to in plasma QUIN determination and the concentration determined ranged from 1.39×10^{-7} to 9.38×10^{-7} M.

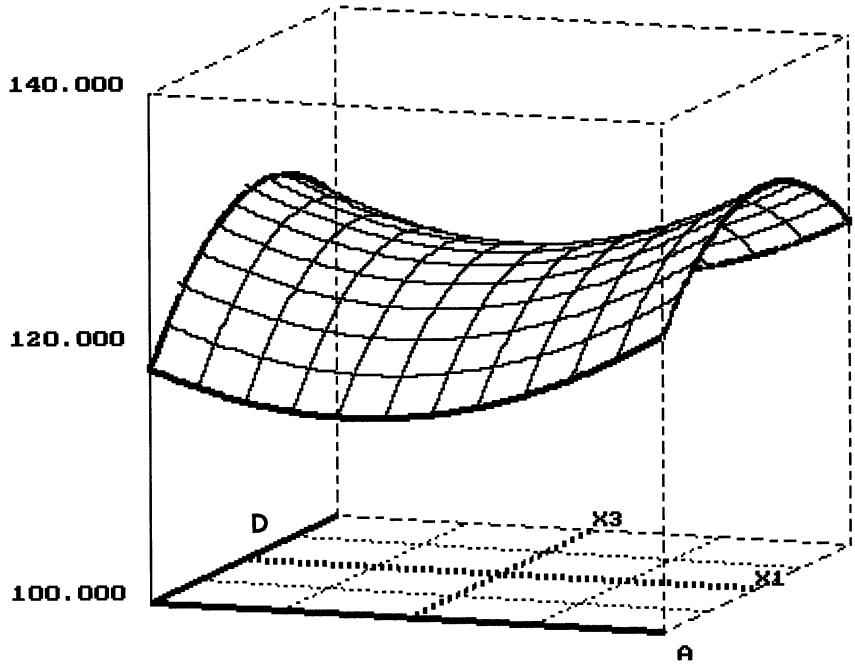

Fig. 6. Response surface for the volume SPE optimization. The retention solvent (A) is plotted against the elution solvent (D), maintaining the wash solvent at the lower level (2 ml).

3.4. Urine analysis

The optimized method was also found to be suitable for QUIN in urine determination although the method was not validated in this biological matrix. The clean-up SPE procedure showed to be optimal also for the urine matrix and QUIN concentration in the analyzed urine samples ranged from 1.5×10^{-5} to 8.3×10^{-5} M, in accordance with the data previously reported [5].

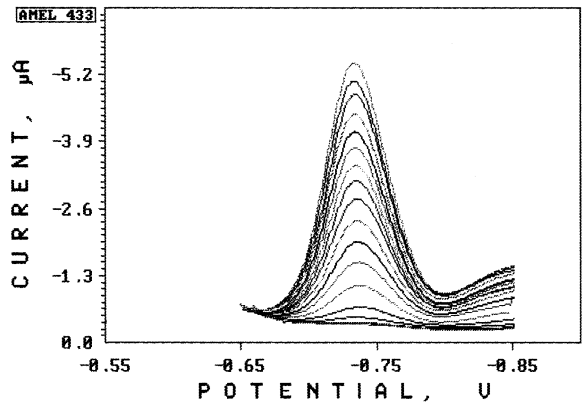

Fig. 7. Differential pulse polarograms of quinolinic acid obtained (after plasma solution polarogram subtraction) for successive additions of the working solution (5.68×10^{-5} M): the first two additions of 15, the third of 50, the fourth of 100 and the successive additions of 250 µl.

4. Conclusions

This paper has shown that a procedure for QUIN determination by differential pulse polarographic reduction can be successfully optimized and validated by means of experimental design tools. However, the methodology can be useful in most other fields of analytical chemistry. In particular practical guidelines to optimize the SPE step are given and the simple approach reported shows that the use of experimental design is advantageous also in sample preparation. In addition, the developed method is easy and fast and can be considered a useful alternative to other methods already established.

Acknowledgements

This work was supported by a grant from the Italian Ministry of the University.

References

[1] T.W. Stone, Pharmacol. Rev. 45 (1993) 310–367.
[2] J. Luthman, E. Vänerman, G. Fredriksson, B. Fornstedt-Wallin, in: G. Allegri Filippini, C.V.L. Costa, A. Bertazzo (Eds.), Recent Advances in Tryptophan Research, Plenum, New York, 1996, pp. 229–239.
[3] I. Nakagawa, A. Sasaki, J. Nutr. Sci. Vitaminol. 23 (1977) 535–548.
[4] G.P. Reynolds, S.J. Pearson, J. Halket, M. Sandler, J. Neurochem. 50 (1988) 1959–1960.
[5] K. Mawatari, K. Oshida, F. Iinuma, M. Watanabe, Anal. Chim. Acta 302 (1995) 179–183.
[6] J.I. Patterson, R.R. Brown, J. Chromatogr. 182 (1980) 425–429.
[7] M.P. Heyes, S.P. Markey, Anal. Biochem. 174 (1988) 349–359.
[8] T. Niwa, H. Yoshizumi, Y. Emoto, et al., Clin. Chem. 37 (1991) 159–161.
[9] E.-L. Sainio, P.H. Hynninen, J. Chromatogr. 420 (1987) 263–274.
[10] P.L. Weber, M. Malis, S.D. Palmer, T.L. Klein, S.M. Lunte, J. Chromatogr. B 697 (1997) 263–268.
[11] A.G. Pozdeeva, E.G. Novikov, Zh. Prikl. Khim. 40 (1967) 213–217.
[12] P.Y. Swamy, G. Veerabhadram, K.S. Sastry, J. Electrochem. Soc. India 36 (1987) 75–77.
[13] P.Y. Swamy, G. Veerabhadram, K.S. Sastry, Z. Phys. Chem. (Leipzig) 27 (1990) 829–836.
[14] S. Furlanetto, S. Pinzauti, P. Gratteri, E. La Porta, G. Calzeroni, J. Pharm. Biomed. Anal. 15 (1997) 1585–1594.
[15] Topic Q2B, Validation of analytical methods: methodology, 3rd Int. Conf. on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH3, Yokohama, Japan, 29 November–1 December 1995.
[16] J. Wieling, H. Dijkstra, C.K. Mensink, et al., J. Chromatogr. 629 (1993) 181–199.
[17] B. Aase, A. Rogstad, J. Chromatogr. A 764 (1997) 223–231.
[18] J.L. Goupy, Methods for Experimental Design, Elsevier, Amsterdam, 1993.
[19] Y. Vander Heyden, D.L. Massart in: M.M.W.B. Hendriks, J.H. de Boer, A.K. Smilde (Eds.), Robustness of Analytical Chemical Methods and Pharmaceutical Technological Products, Elsevier, Amsterdam, 1996, pp. 117–118.
[20] S. Pinzauti, P. Gratteri, S. Furlanetto, P. Mura, E. Dreassi, R. Phan-Tan-Luu, J. Pharm. Biomed. Anal. 14 (1996) 881–889.
[21] A.C. Atkinson, A.N. Donev, Optimum Experimental Designs, Oxford Science Pubblications, Oxford, 1992.
[22] I.E. Frank, R. Todeschini (Eds.), The Data Analysis Handbook, Elsevier, Amsterdam, 1994.
[23] J.C. Miller, J.N. Miller (Eds.), Statistics for Analytical Chemistry, 3rd ed., Ellis Horwood, Chichester, 1993, pp. 110–117.
[24] C. Hartmann, D.L. Massart, R.D. McDowall, J. Pharm. Biomed. Anal. 11 (1994) 1337–1343.
[25] D. Mathieu, R. Phan-Tan-Luu, M. Sergent, in: Criblage et Etude des Facteurs, LPRAI, Marseille, 1996.
[26] R.V. Lenth, Technometrics 3 (1989) 469–473.
[27] S. Furlanetto, C. Tognini, R. Carpenedo, E. La Porta, S. Pinzauti, J. Pharm. Biomed. Anal. (in press).
[28] S. Braggio, R.J. Barnaby, P. Grossi, M. Cugola, J. Pharm. Biomed. Anal. 14 (1996) 375–388.