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Set-up and validation of an adsorptive stripping voltammetric method for kynurenic acid determination in human urine[1]

Sandra Furlanetto [a,*], Clara Tognini [a], Raffaella Carpenedo [b], Enzo La Porta [a], Sergio Pinzauti [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*

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Abstract

Validation of an adsorptive stripping voltammetric method for kynurenic acid determination in urine, was presented. The selection of appropriate validation parameters, the design consideration for evaluation and the problem of endogenous metabolites were discussed. The considered fundamental criteria for assessing the reliability and overall performance of the method in the urine matrix were selectivity, linearity and range, limit of quantitation, accuracy, precision and analyte stability. The intermediate precision was also evaluated by means of a full factorial design. An HPLC method with fluorimetric detection was used as a reference method to assess the accuracy. The analysis in urine required a pH control as pointed out by robustness testing and the found kynurenic acid concentration in daily urine ranged from 5 to 40 μM.

Keywords: Kynurenic acid; Adsorptive stripping voltammetry; Validation; Experimental design; Urine; Bioanalytical method; Quantitation; Endogenous metabolite; Intermediate precision

1. Introduction

Bioanalytical methods must be validated prior to use to engender confidence in the results generated. The evaluation of the quality of analytical methods and results is an important process to determine whether a prospective analytical method meets the needs of a particular project. Validation can be broken into four steps: (1) identification of appropriate validation parameters; (2) design of experiments for parameter evaluation; (3) determination of acceptable method performance for each parameter; and (4) translation of the results into the method procedure. As regards bioanalytical method validation, this requires a prevalidation step with standard solutions, followed by the validation of the method in

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

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the matrix of interest. Reconstituted samples, prepared by adding standards to the biological matrix of interest, are assayed and the relevant parameters that describe the method performance are determined.

In previous work [1] we described the optimization by experimental design and prevalidation of an adsorptive stripping voltammetric (AdSV) method for kynurenic acid (KYNA) determination. KYNA (Fig. 1) is an endogenous triptophan metabolite of considerable biological and clinical significance, present in mammalian biological fluids and the central nervous system and it is an antagonist of ionotropic glutamate receptors [2]. In pharmacological studies, the determination of KYNA in biological fluids is important for studying the behaviour of new inhibitors of kynurenine hydroxylase useful in pathogenic events where an excessive stimulation of ionotropic glutamate receptors has been suggested [3,4]. Since KYNA, human daily urine excretion has been reported to be about 8–13 μmol [5,6], our optimized adsorptive stripping voltammetric method, with a LOQ of 2.5×10^{-9}M [1], seemed to be suitable for the determination of KYNA in urine. Determination of KYNA in urine required a pH control as pointed out by the robustness testing [1]. The validation of the method for KYNA determination in urine was the principal goal and is reported in this paper.

2. Experimental

2.1. Materials

Kynurenic acid was purchased from Sigma (St. Louis, MO) and stored at 4°C. Other standards used were picolinic acid (Sigma) nicotinic acid (Sigma) and quinolinic acid (Sigma). All chemicals and reagents used were of analytical-reagent grade with no further purification. Potassium chloride supporting electrolyte (0.01 M) was prepared by diluting 3 M potassium chloride (Metrohm, Herisau, Switzerland) with water and adjusting the pH to 2.6 with 0.1 M hydrochloric acid. Ultrapure reagent-grade water was obtained with a Milli-Q system (Millipore/Waters, Milford, MA).

2.2. Urine samples

Human urine was collected over 24-h intervals and stored at 4°C until analyzed within 6 h. For the validation study urine samples were collected and immediately analyzed.

The AdSV and HPLC analyses, were carried out with urine solution obtained by diluting urine samples 20-fold with water.

2.3. Equipment

Voltammetric experiments were performed 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. The 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, Universitè de Marseille III, France).

The HPLC apparatus consisted of a Perkin Elmer 250 isocratic pump with a syringe loading sample injection valve (model 7125 Rheodyne, Berkeley, CA). A reverse-phase column (Spherisorb ODS2-10μm, 25 cm, 4.6 mm) was used. The detector used was a fluorimeter (Perkin-Elmer model LC 240).

2.4. AdSV analysis

An accurately weighed amount of KYNA (3 mg) was dissolved in 100 ml of water and the

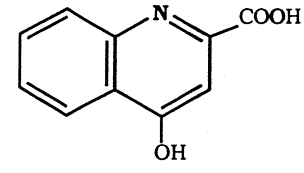

Fig. 1. Kynurenic acid chemical structure.

resulting standard solution was stable for at least 4 days at 4°C. The KYNA working solutions were prepared daily by diluting the standard solution 20-fold with water.

Working solution of picolinic, nicotinic and quinolinic acid were prepared in water at a concentration level of about 1.5×10^{-3} mg ml^{-1}.

The adsorptive stripping procedure was carried out as follows: 10 ml of KCl (0.01 M pH 2.6) were transferred into the voltammetric cell. To the cell 250 μl of urine solution was added together with 30 μl of 3 M HCl to adjust the pH to 2.6. The resulting solution was deareated by bubbling nitrogen-free oxygen for 10 min in the first cycle and 30 s for each successive cycle. The accumulation step on the hanging mercury drop electrode was performed at 0 V for 50 s, stirring the solution at 200 rev min^{-1} with a magnetic stirrer. After an equilibrium time (10 s) a cathodic differential pulse potential scan was applied between -0.5 and -1.1 V with the following optimized settings: scan rate 40 mV s^{-1}; pulse amplitude -60 mV; drop size 40 arbitrary units (a.u.). The voltammetric cycle was repeated twice with a new drop for each analysed solution; the mean of these voltammograms was obtained.

The determination of KYNA in urine was carried out by means of the standard additions method (two additions) and each addition consisted of 100 μl of KYNA working solution. The peak height was automatically measured by the software after setting two reference potentials at the beginning and at the end of the peak and was used to quantify KYNA.

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

For the linearity study the calibration plot was obtained using as a blank 10 ml of supporting electrolyte to which 250 μl of urine solution and 30 μl of HCl 3 M were added. Nine additions of KYNA working solution were then made (the first addition of 15 μl, the second of 35 μl, the third of 100 μl and successive additions of 250 μl). The urine solution voltammogram was considered as a blank and subtracted from the others.

2.5. HPLC analysis

An accurately weighed amount of KYNA (20 mg) was dissolved in 20 ml of water to obtain the 5 mM standard solution daily. KYNA HPLC working solutions (1 mM) were prepared by diluting the standard solution 5-fold with water.

A reverse-phase column was operated isocratically, at room temperature, with 0.05 M sodium acetate buffer (pH 6.2) and 4% acetonitrile as mobile phase. The flow rate was set at 10 ml min^{-1}. The postcolumn derivatizing agent zinc acetate (0.5 M) was delivered at a flow rate of 0.6 ml min^{-1}. The fluorescence was measured with excitation and emission wavelength of 344 and 398 nm, respectively [3].

A 40 μl aliquot of working solution was injected; subsequently, an equal volume of urine solution was injected and evaluated with the postcolumn derivatization method described above. The retention time of KYNA was about 4.5 min. Quantitation of KYNA was achieved by peak height measurements with the external standard method.

3. Results and discussion

3.1. Method set-up

To determine KYNA in urine by voltammetric analysis it is necessary to control the pH of the analyzed solution, the only critical factor with respect to the parameters evaluated as pointed out by the robustness testing [1]. A small change in pH value determines a large change in the observed response. In particular at pH value higher than 3.5 it was observed that the signal due to the KYNA reduction disappeared. Since the pH of the solution resulting from the addition of diluted urine to the supporting electrolyte was about 3.5, it was necessary to adjust the pH to the optimized value of 2.6 in order to quantify KYNA in urine.

The high concentration of HCl (3 M) was chosen in order to minimize the variation of in-cell solution volume. The other parameters did not require any variation with respect to the previously described procedure [1]. Sample handling required only the dilution of the urine samples before voltammetric analysis.

3.2. Validation methodology

The difficulty in validating a bioanalytical method for an endogenous metabolite determination is due to the unknown metabolite amount which is present in the matrix of interest. At the same time, the assay validation has to be the same as other methods, even if the endogenous level of the compound in the biological matrix make it difficult to determine the LOQ and the accuracy [7]. The validation criteria considered here were selectivity, linearity and range, accuracy, precision, limit of quantitation and stability. The precision was evaluated by means of repeatability and intermediate precision.

3.2.1. Selectivity

Some assays are selective; others are specific [8]. Adsorptive stripping voltammetric method is usually selective as it may detect and quantify several compounds, the analytes of interest being identified by their reduction potential [9]. The shift of the reduction potential can be due to several factors, which at times are unknown. Moreover, interfering effects depend on the concentration ratio between the analyte and interfering substances.

Selectivity of the method was tested against some principal triptophan metabolites such as picolinic, quinolinic and nicotinic acids using matrices from six individuals in order to evaluate the interference of unexpected endogenous compounds.

However there is no guarantee that any other studied sample will not contain an interfering compound not observed during method validation [10].

The concentration levels considered for each metabolite were chosen in order to have an in-cell concentration of the analyte similar to the average concentration of KYNA found and higher than the normal value reported in literature [11,12]. The selectivity against quinolinic and picolinic acid was confirmed in all the analyzed matrices. In fact, quinolinic and picolinic acids did not exhibit a reduction peak in the optimized conditions for KYNA.

As regards nicotinic acid, a reduction peak at the potential similar to that of KYNA was evident; however, since the level of nicotinic acid was reported as less than 1/14 of that of KYNA [11], and at such level no signal of nicotinic acid was observed, the determination of KYNA in urine was possible even if the method appears not selective against this metabolite.

3.2.2. Linearity and range

The linearity of an assay method is defined as its ability to elicit results that are directly, or by a mathematical transformation, proportional to the concentration of analyte in samples within a given range [13]. To construct a calibration curve it is necessary to have a blank or a pooled matrix. For endogenous metabolites a blank matrix does not exist, thus the linearity can be assessed with a pooled matrix taking into account the endogenous presence of the analyte. In order to eliminate the current flow due to the KYNA endogenous amount, the urine voltammogram was subtracted and linearity was established in a range from 2.5×10^{-9} to 2.5×10^{-7} M with a R^2 of 0.988 and a cross validated R^2 [14] of 0.937. The large dynamic range found (two orders of magnitude) allows the method to be used to quantify KYNA in urine where large variability could be present.

3.2.3. Accuracy and repeatability

In general the variation in biological matrix makes it difficult to quantify a drug or a metabolite by means of a calibration graph. Multivariate regression techniques can be useful for this kind of problem allowing the analyte in complex matrices to be determined with a multivariate regression model [15]. Unfortunately, determination of endogenous metabolites, such as KYNA, suffers from the absence of blank matrix, thus making the metabolite quantitation by means of a multivariate regression method difficult. Therefore, the

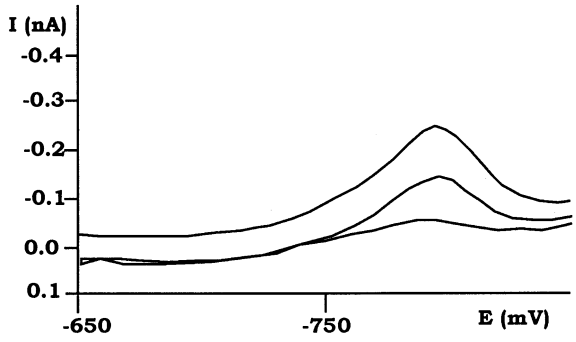

Fig. 2. Adsorptive stripping voltammogram obtained (after urine matrix voltammogram subctraction) to quantify kynurenic acid with standard additions method (2 additions of 100 μl of kynurenic acid solution 1.5×10^{-3} mg ml^{-1}).

quantitation in urine of KYNA required the standard additions method.

After linearity was assessed, accuracy and repeatability (with standard additions method) were tested using as a sample the urine matrix spiked with the working solution and subtracting the voltammogram of urine matrix (Fig. 2). The acceptance criteria for these parameters, at each concentration tested, were: accuracy, $\pm 10\%$ (bias); precision, $\leq 10\%$ (RSD).

The accuracy within-day and repeatability ($n = 5$) were evaluated at the 6×10^{-9}M ($105.8\% \pm 8.2$), 1×10^{-8}M ($96.7\% \pm 5.3$) and 8×10^{-8}M ($103.1\% \pm 4.7$) levels.

The accuracy was also determined comparing the results obtained from five individuals with those of an HPLC reference method with post-column derivatization and fluorimetric detection [3]. The test results were found to be in good agreement as can easily be seen from Table 1. The difference between the mean results of each individual and the accepted true value (HPLC results) was within $\pm 10\%$.

Accuracy of a method expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found [16]. In some respects, such as in the present case in which the endogenous amount is always unknown, it is a difficult parameter to measure. In fact, how closely does a spiked sample reflects the in vivo situation? In addition, the use of a reference method implies that this method is accurate and gives the conventional true value, how is this true? Thus, sometimes, to define an accurate method can be hazardous and the validation of accuracy by means of different methods should be recommended.

3.2.4. Limit of quantitation (LOQ)

To find the LOQ for a method developed for endogenous metabolite, means to analyze a sample with the lowest KYNA concentration with accuracy and precision. In our case, the lowest KYNA concentration level found in the analysed samples was about 5 μM, corresponding to a KYNA in cell concentration of 6×10^{-9}M. Since this concentration level was analysed with accuracy and precision within the above reported acceptance criteria, this value (6×10^{-9}M) could be considered the LOQ of our method in the urine matrix. The use of an acceptance criteria of 15–20% at the LOQ could also be possible, but with some statistical problems [16]. However, the urine matrix depressed the signal making the peak height measurement very difficult to determine at concentration levels lower than 6×10^{-9}M.

3.2.5. Intermediate precision

The intermediate precision is a measure of precision between repeatability and reproducibility. It is obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory [17,18]. Since these parameters influence the response together, it is advisable to study these effects simultaneously. Thus, a Full Factorial Design (FFD) was employed to study these performance parameters. The factors considered were the analyst (analyst 1 and analyst 2), the instrument (same model but with different age; instrument 1 and instrument 2) and the day (day 1 and day 2). The response considered was the determined KYNA concentration and a FFD 2^3 was employed. Each experiment was repeated three times in order to evaluate the experimental error and the analyses were carried out in a randomized order using the working solution according to the experimental plan reported in Table 2. The KYNA in-cell concentration was 2×10^{-8} M. The regression model

Table 1
Comparison between AdSV results and HPLC results obtained from five individuals

	AdSV		HPLC	
Individual	Recovery (μM)	SD ($n=3$)	Recovery (μM)	SD ($n=3$)
1	7.23	0.60	6.89	0.7
2	26.2	1.50	28.9	2.1
3	37.5	2.40	37.6	2.9
4	4.93	0.50	5.12	0.58
5	12.5	0.73	11.7	1.11

assumed was found to be valid and not significant, thus indicating that the intermediate precision of the method was acceptable and no factor considered influenced the response. The RSD was 6.98% ($n=24$) and this value was in the RSD range found in the repeatability study.

3.2.6. Stability

As a part of validation, analyte stabilty in the matrix of interest must be investigated. This information is essential to determine the time that a sample can be left between sampling and freezing or between sampling and analysing. The stability of KYNA in urine at 4°C is about 36 h, after that the voltammetric signal disappears progressively. Storing samples at −20°C a decrease of signal was still observed. Thus, it was decided to collect the sample and directly analyze it voltammetrically.

Table 2
Experimental plan to study intermediate precision

Analyst	Day	Instrument
1	1	1
1	1	1
1	1	1
2	1	1
2	1	1
2	1	1
1	2	1
1	2	1
1	2	1
2	2	1
2	2	1
2	2	1
1	1	2
1	1	2
1	1	2
2	1	2
2	1	2
2	1	2
1	2	2
1	2	2
1	2	2
2	2	2
2	2	2
2	2	2

3.3. Quantitation of KYNA in urine

The daily urine of different subjects was collected. The donors were males and females aged between 20 and 65 years. The KYNA concentration ranged from 5 to 40 μM, apart from a case of an abnormal value of 70 μM. It has to be observed that the KYNA concentration ranges widely for differences in diet and metabolism.

4. Conclusion

Bioanalytical methods play a fundamental role in pharmaceutical and pharmacological research; therefore the data must be produced to acceptable scientific standards. For this reason all bioanalytical methods should be properly validated. In particular, the problem of endogenous compounds determination has to be strongly considered during a validation process. In this paper some remarks and guidelines about this problem have been presented. In addition, the AdSV developed method was shown to be reliable for its intended purpose and to be simpler and faster than the HPLC procedure. Thus it can be used in routine

analysis requiring less expensive equipment and less sample handling than the chromatographic method.

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