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Quantification of 4-aminopyridine in plasma by capillary electrophoresis with electrokinetic injection

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ABSTRACT

A rapid and sensitive CE method for the determination of 4-aminopyridine in human plasma using 3,4-diaminopyridine as an internal standard was developed and validated. The analytes were extracted from 0.5-mL aliquots of human plasma by liquid–liquid extraction, using 8 mL of ethyl ether, and injected electrokinetically into capillary electrophoresis equipment. The instrumental conditions were obtained and optimized by Design of Experiments (DOE – factorial and response surface model), having as factors: separation voltage, ionic strength (buffer concentration), pH and temperature. The response variables were migration time, resolution, tailing factor and drug peak area. After obtaining mathematically predicted values for the response variables with best factors combinations, these were reproduced experimentally in good agreement with predicted values. In addition to optimal separation conditions obtained by Design of Experiments, sensitivity was improved using electrokinetic injection at 10 kV for 10 s, and a capillary with 50 cm effective length and 100 μm I.D. The final instrumental conditions were voltage at 19 kV, capillary temperature at 15 °C, wavelength at 254 nm, and phosphate buffer 100 mM, pH 2.5 as the background electrolyte. This assay was linear over a concentration range of 2.5–80 ng/mL with a lower limit of quantification of 2.5 ng/mL. The relative standard deviation for the assay precision was <7% and the accuracy was >95%. This method was successfully applied to the quantification of 4-aminopyridine (4-AP) in plasma samples from patients with spinal cord injury.

1. Introduction

4-Aminopyridine (4-AP) (Fig. 1) is a potassium channel blocker used in the treatment of patients with spinal cord injury (SCI) or multiple sclerosis (MS). There is evidence that 4-AP is a drug with therapeutic value in enhancing neurological function [1–9] and neurotransmission in preserved axons [10–12]. Its mechanism of action involves increasing the safety factor for axonal conduction at demyelinated internodes [13–15].

In Mexico, 4-aminopyridine has been used with very good results in testing safety and efficacy. Patients showed significant improvement in motor function as well as a persistent effect on sensation and independence. However, patients must be carefully monitored for possible arterial vasospasms at doses of 30 mg/day or higher [16]. Hence, information on 4-aminopyridine pharmacokinetics and their correlation with both, drug efficacy and safety will undoubtedly be useful to optimize the therapeutic use of this drug. For this purpose, sensitive, accurate and precise analytical procedures for determination of 4-aminopyridine plasma levels are required.

Several reports on determination of 4-aminopyridine plasma concentrations by high performance liquid chromatography (HPLC) are available. Hayes et al. [17] reported a method which uses a reversed-phase ion-pair assay with UV detection employing liquid extraction. Gupta and Hansebout [18] reported two reversed-phase column liquid chromatographic procedures for determination of 4-aminopyridine in human serum and urine. In the sample treatment, a procedure with solid-phase extraction (SPE) was used, while in the other, a derivatization reaction with subsequent SPE. The mobile phase contained octanesulfonic acid as ion-pairing agent with UV detection.

Capillary electrophoresis (CE) is considered a highly efficient technique that is simple, selective and versatile. Thus, CE may represent a suitable alternative to HPLC in certain cases [19–21]. It has been reported that CE can be used for the analysis of 4-aminopyridine. Sabbah and Scriba have described CE methods for the determination of 3,4-diaminopyridine, 4-aminopyridine and related impurities. In one study [22], they used a capillary of

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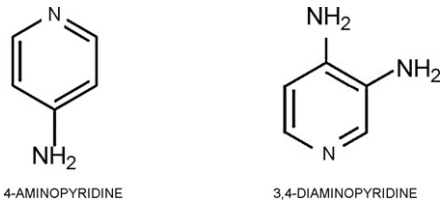

Fig. 1. Chemical structures of 4-aminopyridine and 3,4-diaminopyridine (internal standard).

30 cm effective length, a 50 mM phosphate buffer, pH 2.5 and an applied voltage of 25 kV. In other study [23] they used a capillary of 60 cm effective length, a 100 mM sodium acetate buffer, pH 5.15 and an applied voltage of 20 kV. Yan et al. [24] used a polymer-based monolithic capillary column imprinted with 4-aminopyridine (molecular imprinted polymer) in order to separate this drug and 2-aminopyridine isomers by capillary electrochromatography (CEC).

Although, to our knowledge, CE has not been used for the biopharmaceutical analysis of 4-aminopyridine, the available information suggests that that it can be a suitable option for the determination of 4-aminopyridine plasma concentrations in patients receiving treatment with this drug, as it is an economical and reliable analytical methodology. Hence, the aim of the present study was the development and validation of a simple, sensitive and specific CE method for the determination of 4-aminopyridine, using Design of Experiments (DOE) as a tool for the development and optimization. Design-Expert 6 software [25] was used in this study. In order to improve sensitivity, an electrokinetic sample injection was used [26]. The resulting procedure proved to be suitable for the measurement of 4-aminopyridine plasma concentrations and the determination of its oral pharmacokinetics in patients with spinal cord injury (SCI).

2. Experimental

2.1. Materials and reagents

4-Aminopyridine (>99%) and the internal standard (IS) of 3,4-diaminopyridine (>98%) were obtained from Sigma–Aldrich (Saint Louis, MO, USA). Phosphoric acid (reagent grade) was purchased from J T Baker (Phillipsburg, NJ, USA); ethyl ether (HPLC grade) was purchased from Burdick and Jackson (Muskegon, MI, USA); water (HPLC grade) was obtained from a NANOpure Diamond Water System (Barnstead/Thermolyne, Dubuque, IA, USA). Human plasma was obtained from Medica Sur Hospital (Mexico City). Analysis was performed on uncoated fused-silica capillaries (Beckman-Coulter, Fullerton, CA, USA) with a 50 cm length to detector, 100 μm I.D. and 375 μm O.D.

The phosphate buffer (100 mM) was prepared by thoroughly mixing 1.96 g of H_3PO_4 with 150 mL of water (HPLC grade); pH was adjusted to 2.5 with 0.1N NaOH and diluted to 200 mL with water. All solutions were filtered through 0.45 μm filters.

2.2. Apparatus

Analyses were performed on a P/ACE™ MDQ System (Beckman-Coulter, Fullerton, CA, USA) CE instrument. The eluting peaks were processed with a 32 Karat™ Software, version 5.0 (Beckman-Coulter, Fullerton, CA, USA). Detection was performed on-column at 254 nm. Adjustment of pH was carried out with a Model 555A pH meter using a 617500 solid-state electrode (Thermo Orion, Beverly, MA, USA).

2.3. Preparation of standard solutions

2.3.1. Stock solution of 4-aminopyridine

A stock solution of 4-aminopyridine was prepared by dissolving 12.5 mg in water in a 50 mL volumetric flask (0.250 mg/mL). From this stock solution, a standard solution of 25 μg/mL was prepared by transferring an aliquot of 1 mL of the stock solution into a 10 mL flask and adjusting the volume with water.

2.3.2. Stock solution of internal standard

A stock solution of 3,4-diaminopyridine was prepared by dissolving an accurately weighed amount of 12.5 mg in a 50 mL volumetric flask and adjusting to volume (0.250 mg/mL) A standard solution of 50 μg/mL was prepared by transferring an aliquot of 2 mL of the stock solution into a 10 mL flask and adjusting to volume with water.

2.4. Preparation of sample solutions

2.4.1. Calibration curve

Two solutions of 4-aminopyridine (25 and 5 μg/mL in water) were prepared to obtain the points of the calibration curve. The CE method was evaluated by analysis of seven concentration samples of 4-aminopyridine (2.5, 5, 10, 25, 50, 60 and 80 ng/mL), each sample with an IS concentration of 1000 ng/mL. Each of these calibration points had a total spiked plasma volume of 1 mL. Sample treatment is as mentioned in Section 2.4.2.

2.4.2. Plasma sample preparation

First, 1 mL of plasma (from healthy subjects) plus 20 μL of internal standard (3,4-diaminopyridine 50 μg/mL), 250 μL of sodium hydroxide 0.1N, and 8 mL of ethyl ether were shaken in a test tube for 10 min. The tube was centrifuged at 3000 rpm for 10 min at room temperature (25 °C). The organic phase was transferred to another tube and evaporated to dryness on a water bath at 42 °C under a nitrogen stream. The dry residue was redissolved in 0.3 mL of water and transferred to a 600-μL polypropylene vial (National Scientific, Rockwood, TN, USA). The reconstituted sample was injected electrokinetically at 10 kV for 10 s.

2.5. Method development

After trials, diethyl ether was selected as the liquid–liquid extraction solvent. Electrokinetic injection is a mode of sample introduction for on-line preconcentration of the analytes which compensate a lack of sensitivity of capillary electrophoresis (low injection volume) [26]. For this method, best results were obtained with an applied voltage of 10 kV for 10 s. 3,4-Diaminopyridine was used as internal standard to counterbalance for injection [27] and sample treatment variations, as well as minor fluctuations of the migration times.

Design of Experiments (DOE) is a useful statistical tool that has been applied successfully in the development of analytical methodology by capillary electrophoresis, especially Plackett–Burman (fractional factorial) designs [28], factorial analysis [29,30], central composite designs [31] and Box–Behnken designs [32].

The instrumental conditions were obtained running a complete factorial design 2^4 with two replicates, including central points and a D-optimal design (response surface model), having as factors: separation voltage (10 and 30 kV, central point: 20 kV), ionic strength (buffer concentration 20 and 100 mM, central point: 60 mM), pH (2.5 and 9.5, central point: 6.0), and temperature (15 and 30 °C, central point: 22.5 °C). The response variables were migration time, resolution, tailing factor and drug area.

2.6. Analytical system

CE was performed in free solution using uncoated, fused-silica capillaries with a total length of 57 cm and a 50 cm length to detector, 100 μm I.D. and 375 μm O.D. The analysis time was set at 12 min, 0.5 min purge time with 0.1N sodium hydroxide, 1 min purge with pure water followed by 1 min of equilibration time with phosphate buffer, 100 mM, pH 2.5 (the background electrolyte). The run voltage was set at 19 kV (positive), and the column temperature was controlled at 15 °C. The sample was injected using electrokinetic injection at 10 kV for 10 s. The eluting peaks were processed using 32 Karat™ Software, version 5.0 (Beckman-Coulter, Fullerton, CA, USA), based on peak area.

2.7. Validation

The validation parameters were selectivity, linearity, sensitivity (lower limit of quantification), accuracy, precision and the recovery and stability in human plasma, in accordance with the FDA Guidance for industry [33] and Mexican regulation [34].

Selectivity was studied by comparing the electropherograms of six different batches of plasma obtained from six subjects (healthy subject plasma obtained from the Blood Bank of Médica Sur Hospital, accredited under ISO 15189:2003), with the plasma samples having been spiked with 4-aminopyridine and internal standard. Selectivity also was tested with over-the-counter (OTC) drugs or compounds: acetylsalicylic acid, paracetamol, caffeine and theobromine.

The linearity of the CE method was evaluated by analysis of seven concentration samples of 4-aminopyridine (2.5, 5, 10, 25, 50, 60 and 80 ng/mL), each sample having a concentration of 1000 ng/mL of IS. The area ratio of the drug to internal standard was evaluated for each concentration, plotting these values versus the concentration of 4-aminopyridine. The calibration curves were constructed by least-squares linear regression.

The lower limit of quantification (LLOQ) for 4-aminopyridine was defined as the lowest concentration giving an acceptable accuracy (80–120%), and sufficient precision (within 20%). This was verified by the analysis of six replicates.

Recovery from human plasma samples was evaluated in triplicate for three concentrations of 4-aminopyridine (20, 30 and 40 ng/mL), the response for each level being compared with that from the corresponding standard solution.

The repeatability (intra-assay precision and accuracy) of the method was evaluated by quintuplicate analyses on the same day of three different concentrations of 4-aminopyridine (20, 30 and 40 ng/mL). The reproducibility (inter-assay precision and accuracy) was evaluated in triplicate for three different concentrations of 4-aminopyridine (20, 30 and 40 ng/mL) on three consecutive days. The percentage accuracy was expressed as (mean observed concentration)/(nominal concentration) × 100, and the precision was expressed as the relative standard deviation (RSD, %).

The stability of 4-aminopyridine in human plasma (from healthy subjects, *vide supra*) was assessed by analyzing three replicates at three concentrations (20, 30 and 40 ng/mL) under three conditions: after sample preparation for 48 h at room temperature, after three freeze–thaw cycles, and after long-term storage for 180 days at −70 ± 5 °C. The concentrations obtained were compared with the nominal values of the quality control (QC) samples. The stabilities of stock solutions of 4-aminopyridine and IS were evaluated after a week under refrigeration (2–8 °C) by comparison with a freshly prepared solution at the same concentration.

2.8. Clinical application

Patients with spinal cord injury were eligible for the study if they met the following criteria: at least 1.5 years of tetraplegia or paraplegia, age 18–60 years, medically stable and able to breathe independently, stable neurologic deficits for more than 90 days before the study, no history of epilepsy and electroencephalogram without epileptic activity, and paralyzed extremities without passive limitations (healthy joints). Two patients with spinal cord Injury who were receiving 4-aminopyridine by prescription of their treating physician participated in this study, after giving written informed consent. The first patient was a male of 55 years of age, 74 kg of weight and 179 cm of height with a cervical lesion at the C6–C7 level. The second patient was a male of 19 years of age, 85 kg of weight and 185 cm of height with a thoracic lesion at the T10–T11 level. The protocol was accepted by the institutional review board of the Instituto Mexicano del Seguro Social (Mexico City) and was carried out according with the Recommendations of the Declaration of Helsinki [35], the Rules of Good Clinical Practice [36] as well as with the Mexican General Law on Health [37]. Exclusion criteria were, drug or alcohol abuse and any abnormalities detected in clinical exploration or laboratory screening values. Both patients exhibited hepatic and renal function test results within normal values. After an overnight fast, subjects were given a 4-aminopyridine 10 mg oral capsule (gelatin capsules containing the drug and microcrystalline cellulose as the excipient) with a glass of water. Blood samples (about 8 mL) were collected via a cannula inserted in the cubital vein before and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 4, 6, 8, 12, and 24 h after administration. With a dose of 20 mg (a second 10 mg capsule was administrated 6 h after the first one), blood samples were collected before and at 0.25, 0.5, 1, 1.5, 2, 3, 6, 6.25, 6.5, 7, 7.5, 8, 9 and 12 h after administration. Blood samples were immediately centrifuged at 3000 rpm for 10 min at room temperature, and plasma was stored at −70 ± 5 °C until analysis.

3. Results and discussion

3.1. Method development

After trials, 3,4-diaminopyridine was selected as internal standard (IS) because is an analog compound and to compensate not only for sample preparation variations, but also to diminish the electrokinetic injection variations [27].

A central issue in the development of this analytical method was sensitivity because a maximum lower limit of quantification (LLOQ) of 5 ng/mL was required in order to obtain proper drug-plasma profiles (a maximum plasma concentration C_{max} of about 50 ng/mL with a 10 mg dose) [17]. Fig. 2 (from the complete factorial design 2^4 with two replicates and central points) shows that the highest drug peak area found is at pH 2.5 and at a voltage of around 10 kV. After obtaining mathematical predicted solutions for the response variables (migration time of 4-aminopyridine of about 8 min, resolution with respect to internal standard of about 3, tailing factor of about 1, and maximum area of 4-aminopyridine) with best factors combinations, these were reproduced experimentally in good agreement with predicted values.

The final selected instrumental conditions were: voltage at 19 kV, capillary temperature at 15 °C, wavelength at 254 nm, phosphate buffer 100 mM at pH 2.5. Migration times (M_t) were about 8 and 9 min for 4-aminopyridine and 3,4-diaminopyridine, respectively. Alternative instrumental conditions predicted by mathematical solutions of the designs (all of them at a wavelength at 254 nm) were found and confirmed experimentally with good agreement; all alternative instrumental conditions gave equivalent results.

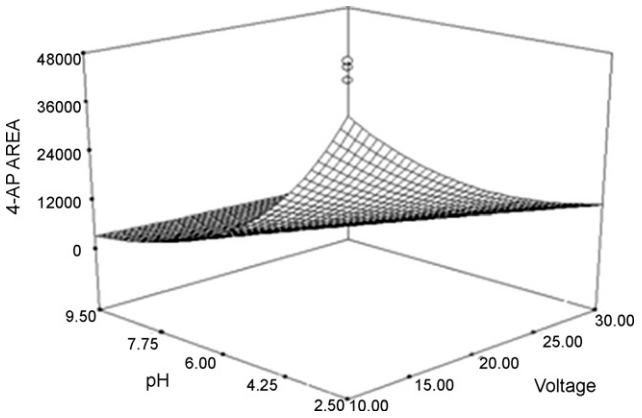

Fig. 2. Effect of pH and voltage in 4-aminopyridine area in proposed CE method. Analytical conditions according to DOE.

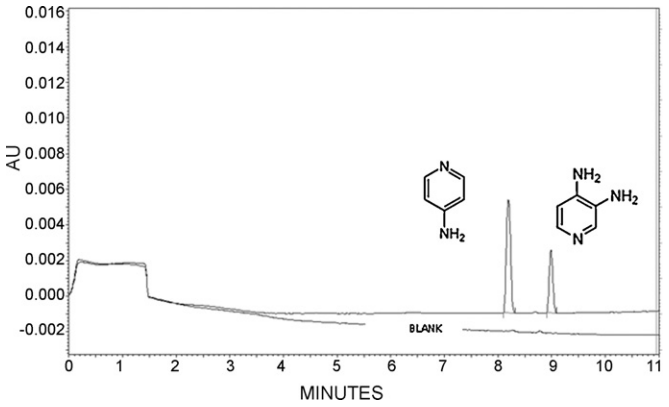

Fig. 3. Typical electropherograms in proposed CE method. Voltage at 19 kV, capillary temperature at 15 °C, wavelength at 254 nm, and phosphate buffer 100 mM pH 2.5.

Alternative solutions were: (a) voltage at 15.8 kV, capillary temperature at 24.6 °C, phosphate buffer 100 mM at pH 2.5, (b) voltage at 15 kV, capillary temperature at 27 °C, phosphate buffer 100 mM at pH 2.5, (c) voltage at 18.5 kV, capillary temperature at 17.4 °C, phosphate buffer 100 mM at pH 2.5.

Under these conditions, the analytical system resolved 4-aminopyridine in the presence of the internal standard (3,4-diaminopyridine) and the unknown component of human plasma. The sample preparation consisted of liquid–liquid extraction with ethyl ether. The solvent was evaporated to dryness. The dry residue was redissolved in water and injected into the electrophoresis system.

The CE method efficiently resolved 4-aminopyridine from the internal standard (3,4-diaminopyridine) and human plasma components. Some samples were spiked with the frequently used OTC medications acetylsalicylic acid, paracetamol, caffeine and theobromine. The presence of these compounds in the samples did not yield any appreciable peak. Design of Experiments (DOE) proved to be a useful tool for helping to find appropriate instrumental conditions, especially in improving sensitivity for this drug, where a lower limit of detection of 500 pg/mL was obtained.

In the development of this analytical methodology by CE, we used a factorial design 2^4 with two replicates and central points, and a D-optimal design (response surface model), obtaining good results, comparable to those reported by Hillaert and Van den Bossche [29] and Capella-Peiró et al. [30] with a 3^2 full factorial design, a central composite design reported by Gil et al. [31], and a Box–Behnken design reported by Hows et al. [32]. The responses variables: migration time of 4-aminopyridine (about 10 min), resolution (about 3, with respect to internal standard), tailing factor (of about 1), and maximum area of 4-aminopyridine, were predicted with best factors combinations (separation voltage, ionic strength (buffer concentration), pH and temperature) and reproduced with very good agreement with experimental values. Another three equivalent instrumental combinations were found and are reported in Section 3.1.

Sabbah and Scriba [22,23] reported two CE methods for quantification of 3,4-diaminopyridine and 4-aminopyridine: separation from related substances and isomeric aminopyridines, respectively. The experimental conditions for the method reported in reference [22] were 50 mM phosphate buffer, pH 2.5 and an applied voltage of 25 kV, while for the method reported in reference [23] the experimental conditions were 100 mM sodium acetate buffer, pH 5.15 and an applied voltage of 20 kV. These experimental results were included in the range of the factors of our experimental designs, where final results of 100 mM phosphate buffer, pH 2.5 and an applied voltage of 19 kV were obtained.

Sabbah and Scriba [22] reported a LLOQ of 2 μg/mL for 4-aminopyridine, using a capillary of 30 cm effective length and 50 μm I.D. Sample solutions were introduced at the anodic end of the capillary at a pressure of 0.5 psi for 3 s, with UV detection at 210 nm. We reproduced their results. However, the requirement was to obtain a maximum LLOQ of 5 ng/mL in order to quantify the entire plasma profile, with an expected C_{max} of about 50 ng/mL (at a 10 mg dose). Sensitivity was thus improved greatly (LLOQ of 2.5 ng/mL, and LLOD of 500 pg/mL) using electrokinetic injection [26] at 10 kV for 10 s, with a capillary of 50 cm of effective length and 100 μm I.D. in proposed CE method. The protoned analytes were concentrated by voltage during injection. Experimental conditions were found as described, with Design of Experiments (DOE), including a capillary temperature of 15 °C. The selected wavelength was 254 nm.

3.2. Method validation

There were no interfering peaks at migration times of about 8 min for 4-aminopyridine and about 9 min for the IS (3,4-diaminopyridine). Fig. 3 shows typical electropherograms for blank plasma and plasma spiked with 4-aminopyridine at 80 ng/mL.

The calibration curves for human plasma provided reliable responses for 4-aminopyridine. The data were best fitted by a linear equation: $mx + b = y$ in the range 2.5–80 ng/mL; the coefficient of determination (R^2) was 0.996.

The LLOQ was 2.5 ng/mL. At 500 pg/mL, a signal-to-noise ratio higher than 3 was obtained so it could be considered as the lower limit of detection (LLOD).

The extraction recoveries of 4-aminopyridine were 76.1%, 81.1% and 81.9% at 20, 30 and 40 ng/mL, respectively, with a mean recovery of 79.7%.

The repeatability (intra-assay precision and accuracy) of the method, which was determined by analyzing five replicates of QC samples at three concentrations on the same day, is shown in Table 1. The reproducibility (inter-assay precision and

Table 1
Precision and accuracy for analysis of 4-aminopyridine in human plasma in proposed CE method.

Nominal concentration (ng/mL)	Precision (RSD, %)		Accuracy (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
20	6.13	3.41	104.7	107.6
30	5.10	3.83	100.3	105.7
40	5.13	6.12	99.1	96.7

RSD: relative standard deviation.

Table 2
4-AP pharmacokinetics of two patients with SCI after an oral dose of 10 mg. One patient (male, 55 years, 74 kg, 179 cm) presented a cervical lesion at C6–C7, the other patient (male, 19 years, 85 kg, 185 cm) presented a thoracic lesion at T10–T11.

Parameters	SCI subjects results reported by Hayes et al. [17], mean (RSD, %)	Patient with thoracic lesion	Patient with cervical lesion
C_{max} (ng/mL)	52.6 (20.6)	62.20	68.90
t_{max} (h)	1.0 (0.6)	1.00	1.00
$t_{1/2}$ (h)	3.6 (0.6)	3.85	2.73
AUC_{∞} (ng h/mL)	241.6 (40.0)	228.23	215.90

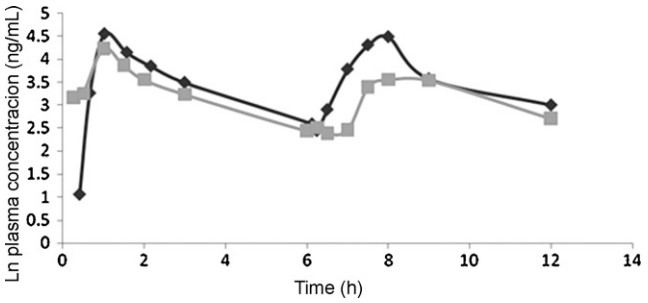

Fig. 4. Plasma profiles of 4-aminopyridine in two patients with spinal cord injury after two oral dose of 10 mg (a second 10 mg capsule was administrated 6 h after the first one). Rhombic = patient with thoracic lesion, squares = patient with cervical lesion.

accuracy) was evaluated in triplicate for three different concentrations of 4-aminopyridine on three consecutive days (Table 1). The intra- and inter-assay accuracies were 99.1–104.7% and 96.7–107.6%, respectively. The relative standard deviation for the intra- and inter-assay precision were 5.1–6.1% and 3.4–6.1%, respectively.

The drug was stable after three freeze–thaw cycles, after long-term storage for 180 days at $-70 \pm 5\,^{\circ}C$, and after sample preparation for 48 h at room temperature (in the autosampler); the stock solutions of 4-aminopyridine and IS were stable for a week under refrigeration (2–8 °C); the results of stability samples were precise and accurate (data are not shown).

3.3. Clinical application

This is the first CE method reported for the quantification of 4-aminopyridine in human plasma. Plasma samples from two patients with spinal cord injury treated with 4-aminopyridine were analyzed using this procedure. Pharmacokinetic parameters are shown in Table 2. As it can be appreciated, the pharmacokinetic parameters were similar to those previously reported using HPLC by Hayes and coworkers [17]. Fig. 4 shows plasma profiles of two patients after administration of two doses of 10 mg of 4-aminopyridine. Hence, the procedure here described has demonstrated to be suitable for pharmacokinetic analysis in patients receiving 4-aminopyridine.

4. Conclusions

A CE method for the quantification of 4-aminopyridine in human plasma was developed using Design of Experiments (DOE) and validated with regard to selectivity, linearity, recovery (%), lower limit of quantification (LLOQ), accuracy, precision and stability. The assay is accurate, precise, rapid and selective. It thus represents a simple, practical and economical option for clinical pharmacokinetic studies in patients treated with medications containing 4-AP as the active substance.

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