Journal of Chromatography B, 878 (2010) 290–294

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

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

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ARTICLE INFO

Article history:
Received 3 September 2009
Accepted 15 November 2009
Available online 18 November 2009

Keywords:
4-Aminopyridine
3,4-Diaminopyridine
Plasma
Capillary electrophoresis
Pharmacokinetics
Pharmacodynamics
Electrokinetic injection

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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doi:10.1016/j.jchromb.2009.11.031

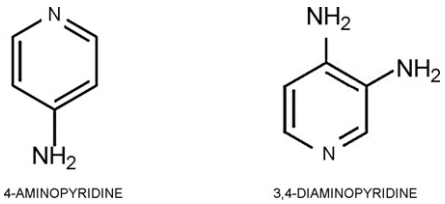

Fig. 1. Chemical structures of 4-aminopyridine and 3,4-diaminopyridine (internal standard).

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.