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Development and validation of a capillary electrophoresis assay for the determination of 3,4-diaminopyridine and 4-aminopyridine including related substances

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Abstract

A capillary electrophoresis (CE) assay has been developed for the quantitation and determination of the impurity profile of the potassium channel blockers 3,4-diaminopyridine and 4-aminopyridine. The compounds were separated from related substances using a capillary of 30 cm effective length, a 50 m*M* phosphate buffer, pH 2.5 and an applied voltage of 25 kV. The assay was validated with respect to specificity, linearity, range, limits of quantitation and detection, precision and robustness. The method allows the detection and quantitation of impurities at the 0.05% level. The feasibility of the assay was demonstrated by analyzing a commercial sample of 3,4-diaminopyridine. All known related substances could be detected in this sample with the present CE method. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Validation; Pharmaceutical analysis; Diaminopyridine; Aminopyridine; Pyridines

1. Introduction

4-Aminopyridine and 3,4-diaminopyridine (Fig. 1) are potassium channel blockers that are effective drugs in improving neuromuscular transmission [1]. Both drugs have been evaluated for the treatment of neuromuscular diseases such as multiple sclerosis. None of the compounds is an approved drug in Europe or North America but 3,4-diaminopyridine has received the status of an orphan drug especially in the therapy of the Lambert–Eaton myasthenic syndrome [2–4]. The German National Formulary (DAC/NRF) lists a monograph of 3,4-diaminopyridine capsules [5]. However, to the best of our knowledge no Pharmacopoeia describes a monograph of the compounds with regard to their identification, purity control and assay. Several papers report the analysis of 4-aminopyridine [6,7] and 3,4-diaminopyridine [8,9] in biological fluids by high-performance liquid chromatography (HPLC).

Capillary electrophoresis (CE) is generally considered a highly efficient technique that is simple, selective and versatile and well capable of analyzing simultaneously both the level of the main component as well as closely related substances. CE has been proven as an alternative to HPLC or thin-layer chromatography (TLC) for the quantitation of compounds and the determination of drug related impurities [10–16]. Quality control examples have been

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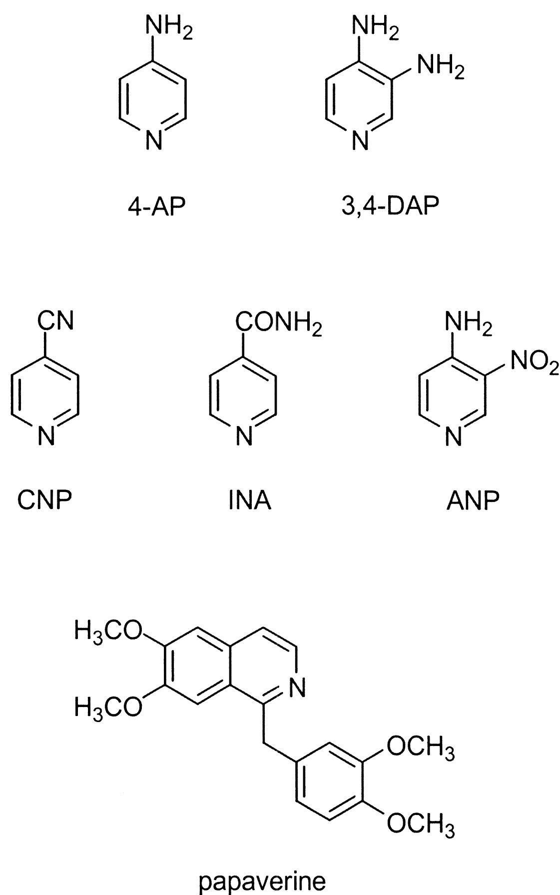

Fig. 1. Structures of the pyridine derivatives and the internal standard papaverine. 4-AP, 4-Aminopyridine; 3,4,-DAP, 3,4-diaminopyridine; CNP, 4-cyanopyridine; INA, isonicotinic acid amide; ANP, 4-amino-3-nitropyridine.

reported where HPLC has been replaced by CE as the method of choice [17].

The objective of this work was the development and validation of an analytical method by CE that allows the simultaneous detection and quantitation of 3,4-diaminopyridine and related compounds. Synthetic impurities include 4-cyanopyridine, isonicotinic acid amide, 4-aminopyridine and 4-amino-3-nitropyridine [18]. Thus, 4-aminopyridine may be evaluated with the same assay. The method was applied to the analysis of commercially available drug substances.

2. Materials and methods

2.1. Materials

4-Amino-3-nitropyridine was a gift from Rüttgers Organics (Mannheim, Germany). 4-Aminopyridine, 3,4-diaminopyridine, 4-cyanopyridine, isonicotinic acid amide and papaverine hydrochloride were obtained from Aldrich (Deisenhofen, Germany) at the purest grade available and used without further purification. Sodium dihydrogenphosphate and phosphoric acid (both analytical-reagent grade) were from E. Merck (Darmstadt, Germany). All buffers and solutions were prepared in deionized, double-distilled water. Stock solutions of the compounds were prepared in the run buffer and stored at −20°C. Fresh working solutions were prepared daily. All solutions were filtered (0.45 μm) and degassed by sonication.

2.2. Apparatus and methods

All experiments were performed on a Beckman P/ACE 5510 instrument (Beckman Coulter, Unterschleissheim, Germany) equipped with a diode array detector at 20°C using 50 μm I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). The effective length of the capillaries was 30 cm, the total length was 37 cm. UV detection at 210 nm was performed at the cathodic end. Sample solutions were introduced at the anodic end at a pressure of 3447.38 Pa (0.5 p.s.i.) for 3 s. Separations were performed in 50 m*M* sodium phosphate buffer, pH 2.5. The pH was adjusted using 100 m*M* H_3PO_4. The applied voltage was 25 kV resulting in a current of 62 μA. A new capillary was conditioned by rinsing with 100 m*M* H_3PO_4 for 20 min, water for 20 min and finally by the run buffer for 20 min. Between analyses the capillary was rinsed with 100 m*M* H_3PO_4 for 1 min followed by run buffer for 2 min. The concentration of the internal standard (papaverine hydrochloride) was 140 μg/ml. The run buffer was replaced every 50 injections. All data were calculated from corrected peak areas as given by the integration software (Beckman P/ACE Station, version 1.2).

3. Results and discussion

3.1. Method development

The present assay was primarily developed for the analysis of 3,4-diaminopyridine but can also be used for 4-aminopyridine. The structures of the analytes are summarized in Fig. 1. The compounds vary considerably in their pK_a values with 4-aminopyridine and 3,4-diaminopyridine being the most basic compounds and 4-cyanopyridine the most acidic compound (Table 1). Thus, initial experiments were performed in the pH range 2–4 as 4-cyanopyridine will be largely unprotonated above pH 4–5 and can therefore be expected to migrate unseparated with the electroosmotic flow (EOF). Using a capillary with an effective length of 30 cm with a 50 m*M* phosphate buffer, pH 2.5, and an applied voltage of 25 kV resulted in a good separation of all compounds including the internal standard, papaverine, within 7 min (Fig. 2). These conditions were used for the validation procedure.

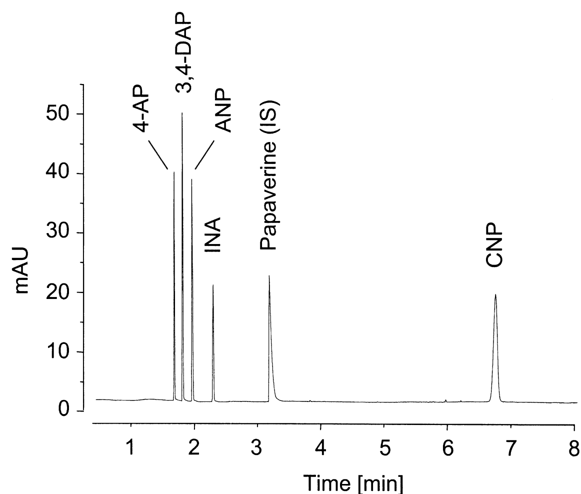

Fig. 2. Electropherogram of standards. Conditions: 37 cm (30 cm effective length)×50 μm I.D. capillary, 50 m*M* phosphate buffer, pH 2.5, 25 kV, 62 μA. 4-AP, 4-Aminopyridine; 3,4-DAP, 3,4-diaminopyridine; CNP, 4-cyanopyridine; INA, isonicotinic acid amide; ANP, 4-amino-3-nitropyridine, papaverine (I.S., internal standard).

3.2. Validation

Papaverine (Fig. 1) was used as internal standard (I.S.). As no related pyridine derivative was available several basic compounds were screened and papaverine was selected based on its migration time between isonicotinic acid and 4-cyanopyridine. The use of an I.S. has been generally accepted to be crucial for reproducibility in CE to compensate for injection errors and minor fluctuations of the migration times [11,15,19,20]. The assay was validated with respect to specificity, linearity, range, limits of quantitation and detection, precision and robustness. The terms are defined according to ICH guideline 2QA [21].

Table 1
Literature apparent pK_a values and calibration data of the pyridine derivatives[a]

Compound[b]	Literature pK_a	Range (mg/ml)	Slope	*R*
4-AP	9.15[c]; 9.21[d]	0.002–0.95	10.049±0.342	0.9996±0.0004
3,4-DAP	9.19[c]; 9.05[e]	0.002–1.02	12.032±0.407	0.9988±0.0005
ANP	5.05[c]	0.003–1.32	7.583±0.375	0.9986±0.0008
INA	3.58[d]; 3.55[f]	0.003–1.16	4.591±0.263	0.9991±0.0007
4-CNP	2.19[f]	0.008–1.05	6.040±0.467	0.9979±0.0011

[a] The values are the mean±standard deviation of three experiments.
[b] For abbreviations see Fig. 1.
[c] Ref. [22].
[d] Ref. [23].
[e] Ref. [24].
[f] Ref. [25].

3.2.1. Specificity

4-Aminopyridine may contain 4-cyanopyridine and isonicotinic acid amide as impurities while related substances of 3,4-diaminopyridine include 4-cyanopyridine, isonicotinic acid amide, 4-aminopyridine and 4-amino-3-nitropyridine. Under the applied conditions the main compounds 3,4-diaminopyridine and 4-aminopyridine are well separated from their respective impurities (Fig. 2) allowing the identification as well as quantitation of the pyridine derivatives. Fig. 3B shows a representative electropherogram of 3,4-diaminopyridine at a concentration of 5 mg/ml (concentration as obtained from weighing the compound) spiked with the impurities at the 0.05% level (2.5 μg/ml) compared to an electropherogram of pure reference material (Fig. 3A). In the same way, 0.04% of the related compounds could be detected in a solution of 7.5 mg/ml 4-aminopyridine (data not shown). Thus, the assay allows the detection of at least 0.05% of the related compounds. Concentrations of 3,4-diaminopyridine greater than 7 mg/ml led to peak splitting as a result of concentration overload. These effects may be avoided by using a higher buffer molarity. However, this was not further investigated in the present study.

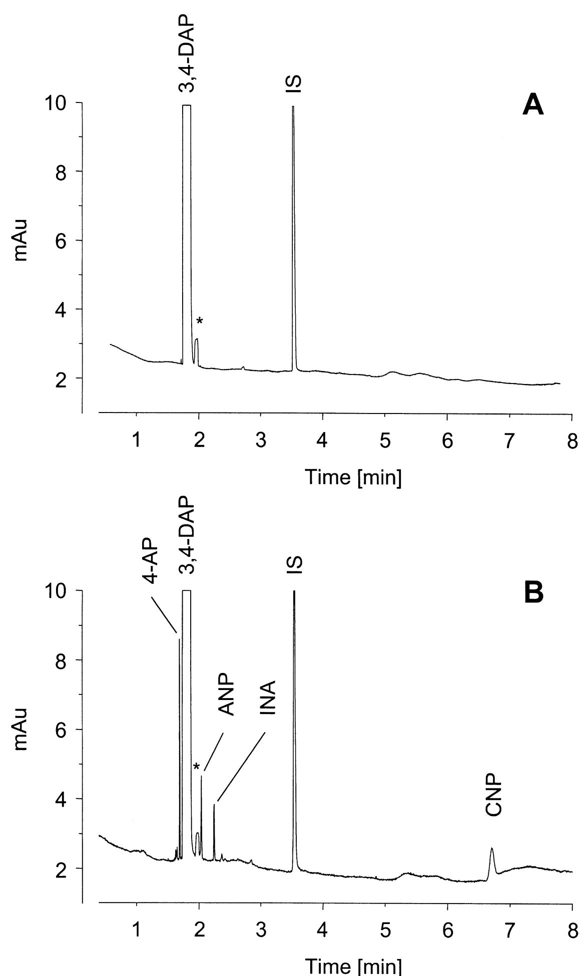

Fig. 3. Electropherogram of 5 mg/ml 3,4-diaminopyridine (A) reference compound and (B) spiked with 0.05% of the related compounds. Conditions and peak labeling see Fig. 2. The asterisk denotes an unknown impurity.

3.2.2. Linearity, range, limits of quantitation and detection

Depending on the pyridine derivative the assay was calibrated in the range of approximately 2 μg/ml to 1.4 mg/ml using the peak area ratio method. Calibration curves were constructed from nine different concentrations. Each concentration was injected five times. The data of the calibration curves obtained from three experiments are summarized in Table 1. Linear relationships with regression coefficients (R) of at least 0.997 were found for all substances. The limits of quantitation determined in a single experiment were 2.0 μg/ml for 4-aminopyridine and 3,4-diaminopyridine, 2.5 μg/ml for 4-amino-3-nitropyridine and isonicotinic acid amide and 3.5 μg/ml for 4-cyanopyridine. The limits of detection defined as signal-to-noise ratio of 3:1 were about 1 μg/ml for 4-aminopyridine and 3,4-diaminopyridine and about 1.5 μg/ml for 4-amino-3-nitropyridine, isonicotinic acid amide and 2.0 μg/ml for 4-cyanopyridine.

3.2.3. Precision

The precision of the assay was investigated with respect to repeatability and intermediate precision. For intra-day precision three concentrations of each compound were analyzed in three independent series on the same day. Within each series every sample was injected five times. For an estimate of the day-to-day precision (inter-day precision) similar samples were analyzed on 3 consecutive days. Each sample was injected 4–5 times. Table 2 summarizes the relative standard deviations (RSDs) of the ratios

Table 2
Intra-day and day-to-day precision of the pyridine derivatives[a]

Compound[b]	Concentration (mg/ml)	Intra-day RSD (%) (n=12)	Day-to-day RSD (%) (n=15)
4-AP	0.002	6.63	7.23
	0.225	1.11	1.49
	0.900	0.99	0.75
3,4-DAP	0.002	7.97	7.47
	0.255	1.37	1.22
	1.020	0.76	1.07
ANP	0.003	8.75	8.97
	0.330	1.08	1.49
	1.320	0.66	1.16
INA	0.003	8.88	7.84
	0.290	1.21	1.39
	1.165	0.89	0.73
CNP	0.255	1.73	1.64
	1.025	1.31	1.93

[a] RSDs of the area ratio of the individual compounds versus the internal standard papaverine are presented. The samples were analyzed in three runs either within 24 h (intra-day) or on 3 consecutive days (day-to-day). Each concentration was injected 4–5 times.
[b] For abbreviations see Fig. 1.

corrected area pyridine versus corrected area of the internal standard papaverine. Generally acceptable repeatability of the areas within 1 day and day-to-day was observed. At the lowest concentration the RSD of the area ratio was between 6.63 and 8.97% while at higher concentrations RSDs of about 1.93% or less were found except for 4-cyanopyridine.

Data of the absolute and relative migration times obtained in a series of 45 consecutive injections within 24 h are summarized in Table 3. Generally, acceptable repeatability expressed as the RSD is observed. The RSDs varied between 1.28 and 1.77% except for 4-cyanopyridine which displayed a RSD of 6.44%. Improvement of the precision was obtained by applying the migration time relative to an I.S. as reported by others [11,19,20]. An RSD of 1.05% or less was found for the relative migration times of the pyridines relative to the internal standard papaverine except for 4-cyanopyridine at 4.32%. Day-to-day precision of the migration times obtained for a total of 39 injections on 3 consecutive days was somewhat lower compared to the intra-day repeatability (Table 3). The RSDs ranged between 1.56 and 4.76% for the absolute migration times and between 1.20 and 4.36% for the relative migration times. The relatively poor general precision of the migration time of 4-cyanopyridine may be due to the fact that this compound is the least basic pyridine derivative. The pK_a is close to the pH of the run buffer. Thus, small changes of the operational parameters will have largest effect on this compound as reflected by relatively high RSD.

The influence of the capillary on the repeatability of the relative migration times of the assay was estimated using four different capillaries from two different batches. Prior to the analyses each capillary was pretreated as described under Materials and methods by subsequent rinses with phosphoric acid, water and the run buffer. A sample containing 220–250 μg/ml depending on the pyridine derivative was analyzed five times. The RSDs of the individual relative migration times of the pyridines within a single capillary varied between 0.19 and 2.80% with the 4-cyanopyridine exhibiting the largest RSD. The

Table 3
Absolute migration times (t_m), migration times relative to the internal standard papaverine (t_m rel) and RSD analyzed within the same day (intra-day) and on 3 subsequent days (day-to-day)

Compound[a]	Intra-day (n=45)				Day-to-day (n=39)			
	t_m (min)	RSD t_m (%)	t_m rel (min)	RSD t_m rel (%)	t_m (min)	RSD t_m (%)	t_m rel (min)	RSD t_m rel (%)
4-AP	1.71	1.28	0.516	0.95	1.72	1.56	0.516	1.20
3,4-DAP	1.85	1.44	0.558	0.99	1.86	1.72	0.557	1.24
ANP	2.01	1.48	0.606	0.93	2.02	2.05	0.605	1.24
INA	2.33	1.77	0.704	1.05	2.33	2.88	0.704	2.41
CNP	6.88	6.44	2.091	4.32	7.44	4.76	2.204	4.36
Papaverine	3.31	1.46	–	–	3.38	2.90	–	–

[a] For abbreviations see Fig. 1.

Table 4
Relative migration times of the pyridine derivatives upon changes of the buffer pH and applied voltage[a]

Compound[b]	Constant applied voltage 25 kV			Constant pH 2.5		
	pH 2.3	pH 2.5	pH 2.7	23 kV	25 kV	27 kV
4-AP	0.443 (0.09%)	0.503 (0.23%)	0.500 (0.34%)	0.495 (0.11%)	0.503 (0.23%)	0.505 (0.69%)
3,4-DAP	0.484 (0.17%)	0.543 (0.13%)	0.541 (0.27%)	0.536 (0.12%)	0.543 (0.13%)	0.545 (0.63%)
ANP	0.532 (0.26%)	0.589 (0.12%)	0.588 (0.23%)	0.583 (0.15%)	0.589 (0.12%)	0.592 (0.47%)
INA	0.583 (0.06%)	0.647 (0.14%)	0.692 (0.42%)	0.638 (0.18%)	0.647 (0.14%)	0.650 (0.50%)
CNP	1.903 (1.62%)	1.818 (0.60%)	2.517 (1.35%)	1.829 (0.28%)	1.818 (0.60%)	1.814 (0.87%)

[a] The values are the mean of five injections. RSD of the mean is listed in parentheses.
[b] For abbreviations see Fig. 1.

RSDs calculated over all four capillaries were 3.06%, 3.21%, 3.11%, 5.39% and 4.27% for 4-aminopyridine, 3,3-diaminopyridine, 4-amino-3-nitropyridine, isonicotinc acid amide and 4-cyanopyridine, respectively.

3.2.4. Robustness

The influence of significant changes of the buffer pH (pH 2.3–2.7) and the applied voltage (23–27 kV) was also investigated. These ranges represent variations of ±8% of the experimental standard conditions. Buffer molarity as another possible parameter was not included since no significant effects were observed during method development. A sample containing 45–320 μg/ml depending on the pyridine was analyzed three times at pH 2.2, 2.5 and 2.7 at an applied voltage of 25 kV while the applied voltage was varied from 23 kV to 27 kV at a constant pH of 2.5. Although the absolute migration times of the compounds generally decreased with decreasing buffer pH the relative migration times did not change significantly except for 4-cyanopyridine (Table 4). As stated above, this may be due to the fact that the pH of the run buffer is close to the pK_a of the compound and small changes in the operational parameters, therefore, will have a larger effect on 4-cyanopyridine than on compounds whose pK_a is much larger than the buffer pH as for the other pyridine derivatives investigated. There was no significant effect on the separation efficiency expressed as the selectivity α ($\alpha = t_{m2}/t_{m1}$) upon changing the buffer pH or the applied voltage (data not shown).

Table 5 summarizes the area ratios of the pyridine derivatives versus the internal standard upon variations of the buffer pH and the applied voltage. Changes of the applied voltage between 23 and 27 kV did not effect the area ratios to a great extent. Deviations of maximal 1.9% relative to the ratio at 25 kV (used as reference value) were observed. In contrast, significant changes of the ratios depending on the pH of the run buffer were found. The effects were more pronounced when lowering the pH to 2.3. Generally, lower area ratios translating to lower concentration values were found for all derivatives. The deviations relative to the value at pH 2.5 (used as reference value) ranged between 7 and 10% except for 4-cyanopyridine which exhibited a devia-

Table 5
Peak area ratios of the pyridine derivatives versus the internal standard papaverine upon changes of the buffer pH and applied voltage[a]

Compound[b]	Constant applied voltage 25 kV			Constant pH 2.5		
	pH 2.3	pH 2.5	pH 2.7	23 kV	25 kV	27 kV
4-AP	1.882 (0.96%)	2.001 (0.71%)	1.998 (0.56%)	2.012 (0.35%)	2.001 (0.71%)	1.995 (0.26%)
3,4-DAP	2.824 (1.18%)	2.943 (0.22%)	2.941 (0.39%)	2.931 (0.52%)	2.943 (0.22%)	2.941 (0.19%)
ANP	2.337 (1.16%)	2.419 (0.36%)	2.398 (0.40%)	2.390 (0.69%)	2.419 (0.36%)	2.404 (0.40%)
INA	1.091 (1.66%)	1.166 (0.43%)	1.162 (0.42%)	1.154 (0.86%)	1.166 (0.43%)	1.169 (0.87%)
CNP	0.163 (3.30%)	0.270 (0.50%)	0.264 (0.79%)	0.265 (0.87%)	0.270 (0.50%)	0.275 (0.49%)

[a] The values are the mean of five injections. RSD of the mean is listed in parentheses.
[b] For abbreviations see Fig. 1.

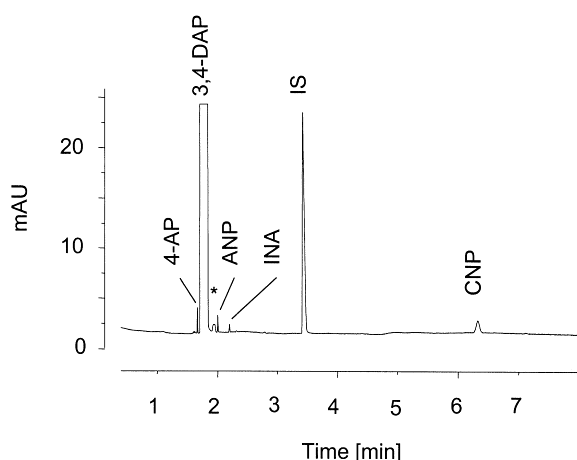

Fig. 4. Electropherogram of a commercial sample of 3,4-diaminopyridine (5.7 mg/ml). Conditions and peak labeling see Fig. 2. The asterisk denotes an unknown impurity.

tion of about 40%. Increasing the pH did not effect the area ratios in the same way. The largest relative deviation at pH 2.7 compared to pH 2.5 was observed for isonicotinic acid amide with 2.9%. Thus, changes in the buffer pH have a larger influence on the assay compared to variations of the applied voltage.

3.3. Application

The assay was applied to the analysis of a commercial sample of 3,4-diaminopyridine. Fig. 4 shows the electropherogram of a solution containing 5.7 mg/ml of the sample (concentration derived from weighing of the compound). All related substances could be detected. The following relative concentrations of the known impurities in 3,4-diaminopyridine were found by the present CE method: 4-aminopyridine 0.04%, 4-amino-3-nitro-ypridine 0.04%, isonicotinic acid amide 0.03%, and 4-cyanopyridine 0.08%. The values are the mean of two injections of the sample.

4. Conclusions

A CE method for the analysis of 4-aminopyridine and 3,4-diaminopyridine including related substances was developed and validated with regard to specificity, linearity, range, limits of quantitation and detection, precision, and robustness. The assay was found to be precise, accurate and robust allowing the quantitation of the compounds as well as the detection of impurities below the 0.05% level. Thus, in agreement with other studies [11–17,19] CE may be a powerful method for the determination and purity control of drug substances.

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