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Validation of a CE assay for the analysis of isomeric aminopyridines and diaminopyridines

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Abstract

A capillary electrophoresis assay for the analysis of aminopyridines and diaminopyridines has been developed and validated. The compounds were separated using a 100 mM sodium acetate buffer at pH 5.15, an applied voltage of 20 kV and a capillary of 60 cm effective length. *N*-(1-naphthyl)ethylenediamine was used as internal standard to compensate for injection errors and minor fluctuations of the migration times. The detection wavelength was set at 240 nm and not optimized for a specific derivative. The assay was validated with respect to specificity, linearity, range, limit of quantitation and detection, precision, and robustness. Within certain limits, the assay also allowed the detection and determination of the other aminopyridine derivatives at the 0.1% level as demonstrated for 3,4-diaminopyridine.

Keywords: Diaminopyridine; Aminopyridine; Capillary electrophoresis; Validation

1. Introduction

Of the six commercially available aminopyridines and diaminopyridines (Fig. 1), two derivatives, 4-aminopyridine and 3,4-diaminopyridine, are effective drugs in improving neuromuscular transmission by blockade of potassium channels [1]. Both drugs have been evaluated for the therapy of neuromuscular diseases, such as multiple sclerosis but none of the compounds is an approved drug in Europe or North America. 3,4-diaminopyridine has received the status of an orphan drug in the USA for the treatment of the Lambert–Eaton myasthenic syndrome [2–4]. The German national formulary (Neues Rezeptur-Formularium) 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. A reversed phase ion pair HPLC assay of the aminopyridine derivatives has been reported [6]. However, it cannot be unequivocally concluded from the data if complete separation of all six compounds was achieved. The analysis of 4-aminopyridine [7,8] and 3,4-diaminopyridine [9,10] in biological fluids by HPLC has also been reported.

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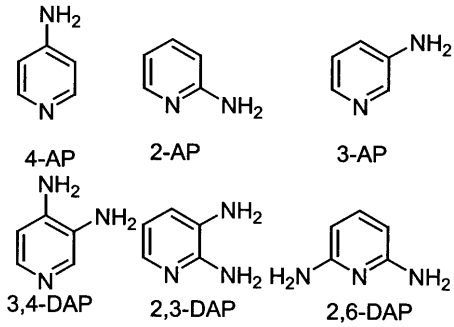

Fig. 1. Structures of the aminopyridine derivatives. 4-AP, 4-aminopyridine; 3-AP, 3-aminopyridine; 2-AP, 2-aminopyridine; 3,4-DAP, 3,4-diaminopyridine; 2,3-DAP, 2,3-diaminopyridine; 2,6-DAP, 2,6-diaminopyridine.

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 proven as an alternative to HPLC or TLC for the quantitation of compounds and the determination of drug related impurities [11–22]. Thus, the objective of this work was the development and validation of an analytical method by CE that allows the simultaneous detection and quantitation of all six aminopyridine derivatives.

2. Experimental

2.1. Materials

2-aminopyridine, 3-aminopyridine, 4-aminopyridine, 2,3-diaminopyridine, 2,6-diaminopyridine, 3,4-diaminopyridine and *N*-(1-naphthyl)ethylenediamine dihydrochloride were obtained from Aldrich Chemical Co. (Deisenhofen, Germany) at the purest grade available and used without further purification. Sodium acetate p.a., acetic acid p.a. and phosphoric acid p.a. were from E. Merck (Darmstadt, Germany). All buffers and solutions were prepared in deionized, double-distilled water. Stock solutions of the compounds and diluted samples were prepared in the run buffer. 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 GmbH, Unterschleißheim, Germany) equipped with a diode array detector at 20°C using 50 μm I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, Arizona, USA). The effective length of the capillaries was 60 cm, the total length was 67 cm. UV-detection at 240 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 100 mM sodium acetate buffer, pH 5.15. The pH was adjusted using 100 mM acetic acid. The applied voltage was 20 kV resulting in a typical current of 31 μA. A new capillary was conditioned by rinsing with 100 mM 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 mM H_3PO_4 for 1 min followed by run buffer for 2 min. The concentration of the internal standard, *N*-(1-naphthyl)ethylenediamine dihydrochloride, was 150 μg/ml. The run buffer was replaced every 25 injections.

3. Results and discussion

3.1. Method development

The structures of the isomeric aminopyridines and diaminopyridines are summarized in Fig. 1. The compounds vary considerably in their pK_a values (Table 1). Initial experiments were performed in 50 mM phosphate buffer in the pH range 2.5–6 so that all compounds were largely protonated and migrated as cations. However, only incomplete separation could be obtained under these experimental conditions. Changing the buffer to a 100 mM sodium acetate buffer, greatly improved the separation. Complete separation was achieved at pH 5.15. Increasing the effective length of the capillary from 50 to 60 cm further improved peak resolution. Thus, using a capillary of 60 cm effective length, a 100 mM sodium

Table 1
Literature apparent pK_a values and calibration data of the aminopyridine derivatives

Aminopyridine[a]	pK_a	Range [mg/ml]	Slope	*R*
4-AP	9.15[b], 9.21[c]	0.0016–1.07	4.525	0.9998
2-AP	6.71[b]	0.0022–0.89	4.599	0.9999
3-AP	6.01[b], 6.14[c]	0.0018–1.05	6.168	0.9996
3,4-DAP	9.19[b], 9.05[d]	0.0016–1.17	5.355	0.9996
2,3-DAP	6.73[b]	0.0025–1.06	3.802	0.9997
2,6-DAP	6.00[b]	0.0015–1.15	7.758	0.9998

[a] For abbreviations see Fig. 1.
[b] Ref. [25].
[c] Ref. [26].
[d] Ref. [27].

acetate buffer, pH 5.15, and an applied voltage of 20 kV, all six aminopyridines and the internal standard, *N*-(1-naphthyl)-ethylenediamine, could be separated within 13 min (Fig. 2). Under these conditions, the electroosmotic flow was recorded at 43.96 ± 0.11 min ($n = 15$). The aminopyridines differ considerably in their UV maxima [6]. Thus, UV detection was carried out at 240 nm, where all six pyridine derivatives displayed acceptable absorbance according to the spectra recorded by the DAD detector. These conditions were used for the validation procedure.

3.2. Validation

N-(1-naphthyl)ethylenediamine was used as internal standard. Numerous studies have shown that the use of an internal standard is crucial for reproducibility in CE in order to compensate for injection errors and minor fluctuations of the migration times [12,16,20–23]. The assay was validated with respect to linearity, range of quantitation, detection, precision, specificity and robustness. The terms are used according to the definition of the ICH guideline 2QA [24].

3.2.1. Linearity, range, limit of quantitation and detection

Depending on the aminopyridine, the assay was calibrated in the range of 1.5–1.17 mg/ml using the peak area ratio method. Calibration curves were constructed from eight to nine different concentrations. Each concentration was injected five times. The data of the calibration curves obtained from three independent experiments are summarized in Table 1. Linear relationships with regression coefficient, *R*, of at least 0.9996 were found for all substances. At the lowest concentration used for the calibration the relative standard deviation (RSD) of the area ratios was 10% or better. This concentration was, therefore, regarded as the limit of quantitation of the present assay, allowing the determination of the pyridines with acceptable accuracy and precision. The detection limit defined as signal to noise ratio of 3:1 was 0.8 μg/ml for 2-aminopyridine and 2,3-diaminopy-

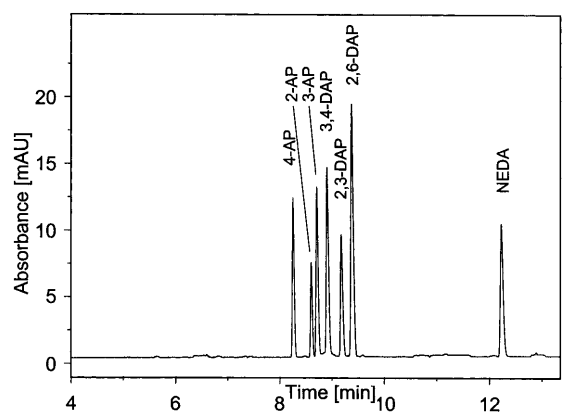

Fig. 2. Electropherogram of standards. Conditions: 67/60 cm capillary, 50 μm I.D., 100 mM sodium acetate buffer, pH 5.15, 20 kV, 31 μA. 4-AP, 4-aminopyridine; 2-AP, 2-aminopyridine; 3-AP, 3-aminopyridine; 3,4-DAP, 3,4-diaminopyridine; 2,3-DAP, 2,3-diaminopyridine; 2,6-DAP, 2,6-diaminopyridine; NEDA, *N*-(1-naphthyl)ethylenediamine (internal standard).

Table 2
Intraday and day-to-day precision of the area ratio pyridine derivative versus internal standard[a]

Compound[b]	Concentration [mg/ml]	Intraday precision		Day-to-day precision	
		Area ratio	RSD (%)	Area ratio	RSD (%)
4-AP	0.013	0.0807	4.04	0.0807	3.97
	0.262	1.344	0.70	1.346	1.07
2-AP	0.011	0.0607	5.2	0.0612	5.93
	0.222	1.143	0.85	1.161	1.70
3-AP	0.013	0.1055	4.20	0.1060	5.06
	0.252	1.760	0.76	1.805	1.58
3,4-DAP	0.013	0.1021	5.02	0.1039	5.21
	0.262	1.597	0.75	1.622	2.07
2,3-DAP	0.012	0.0519	6.27	0.0506	3.93
	0.249	1.029	0.86	1.035	1.73
2,6-DAP	0.014	0.1481	5.45	0.1419	4.58
	0.286	2.469	0.66	2.501	1.99

[a] The samples were analyzed in three runs either within 24 h (intraday) or on three consecutive days (day-to-day). Each concentration was injected five times.
[b] For abbreviations see Fig. 1.

ridine and 0.5 μg/ml for 4-aminopyridine, 3-aminopyridine, 3,4-diaminopyridine and 2,6-diaminopyridine. The quantitation data apply to the present assay with UV detection at 240 nm. The sensitivity of an individual aminopyridine may be further increased by monitoring at the UV maximum of the respective compound. However, the aim of the present study was the development of an assay allowing the simultaneous quantitation of all six pyridines, so it was not optimized for any specific derivative.

3.2.2. Precision

The assay was investigated with respect to repeatability and intermediate precision. Two concentrations of each compound were analyzed in three independent series on the same day (intraday precision) and on three consecutive days (day-to-day precision). Within each series every sample was injected five times. The data of the ratio corrected area of the pyridine derivative versus corrected area of the internal standard are summarized in Table 2. Generally acceptable repeatability of the area ratios within one day and within three days was observed. The RSDs at the lower concentration varied between 3.97% and 6.27%. RSDs of 0.70% to 1.99% were found at the higher concentration. Intraday precision was slightly better than day-to-day precision as expressed in the lower RSDs. Good precision of the absolute migration times was observed for analyses run on the same day or on consecutive days (Table 3). The RSD of the migration times never exceeded 0.60%. The repeatability was further improved by applying the migration time ratio method relative to the internal standard *N*-(1-naphthyl)ethylenediamine. The RSDs of the relative migration times were 0.44% or less (Table 3). Similar observations have been reported in several studies [12,20–23].

3.2.3. Specificity

All six aminopyridines are well separated allowing the quantitation as well as the identification of the compounds. The identification was achieved by either comparison of the absolute or relative migration times, by spiking with the individual compounds or by comparison of the UV spectra when using a DAD detector as the aminopyridines differ considerably in their UV spectra [6].

Table 3
Absolute migration times, t_m, migration times relative to the internal standard (t_m rel) and RSDs of the aminopyridines analyzed within 24 h (intraday) or on three consecutive days (day-to-day)

Compound[a]	Intraday precision ($n = 15$)				Day-to-day precision ($n = 15$)			
	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	8.03	0.29	0.687	0.14	8.04	0.30	0.687	0.13
2-AP	8.36	0.30	0.715	0.13	8.36	0.31	0.714	0.13
3-AP	8.47	0.22	0.725	0.20	8.48	0.20	0.724	0.23
3,4-DAP	8.65	0.33	0.740	0.11	8.64	0.60	0.739	0.44
2,3-DAP	8.91	0.29	0.762	0.15	8.91	0.36	0.762	0.12
2,6-DAP	9.10	0.30	0.779	0.13	9.11	0.43	0.778	0.16
NEDA	11.70	0.40	–	–	11.69	0.48	–	–

[a] For abbreviations see Fig. 1; NEDA, *N*-(1-naphthyl)ethylenediamine.

Fig. 3 shows an electropherogram of 3,4-diaminopyridine at a concentration of 5.7 mg/ml spiked with 0.06% of the other aminopyridines. Except for 3-aminopyridine which comigrates with the major sample component under the standard assay conditions, all compounds may be clearly detected. When a sample of 3,4-diaminopyridine containing 1% of the other compounds was analyzed at pH 5.10, 3-aminopyridine could be detected as a shoulder but reliable integration of the peak was not possible (see inset in Fig. 3). The influences of small variations of the buffer pH are discussed below. Overall, except for 3-aminopyridine, the method is also applicable to test for most of the aminopyridines as potential impurities of 3,4-diaminopyridine at the 0.1% level.

3.2.4. Robustness

The assay was tested for robustness to deliberate small changes in the operating parameters with regard to buffer pH (pH 5.10–5.20), buffer molarity (90–100 mM), applied voltage (19–21 kV), and detection wavelength (238–242 nm). Only one parameter was changed in the experiments at a time. A sample containing between 250–315 μg/ml depending on the pyridine derivative was analyzed five times. The migration times relative to the internal standard under the various conditions are summarized in Table 4. As the detection wavelength does not effect the migration times these data were not included. Despite small changes in the absolute migration times the relative migration times proved to be quite stable towards the investigated variations of the experimental conditions. The largest deviation relative to the standard operating conditions (defined as 100%) was 1.76%. However, the buffer pH effected peak resolution. Electropherograms of the pyridine derivatives at pH 5.10 and 5.20 are shown in Fig. 4. Compared to the electrophero-

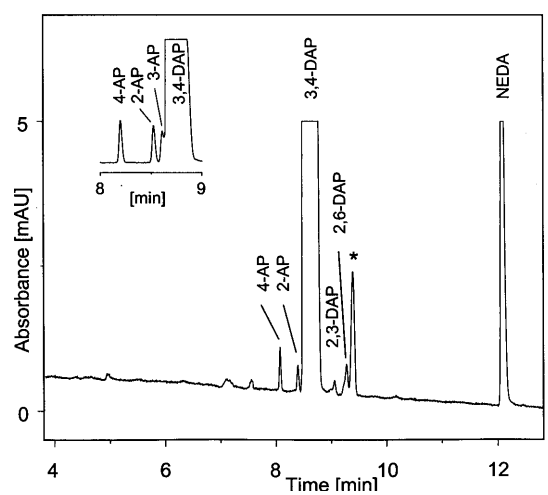

Fig. 3. Electropherogram of a solution of 5.7 mg/ml 3,4-diaminopyridine containing 0.06% of the other aminopyridine derivatives. Conditions and abbreviations see Fig. 2. The asterisk denotes an unknown impurity. The inset shows an electropherogram of 3,4-diaminopyridine spiked with 1% of the aminopyridines run at pH 5.10.

Table 4
Robustness of the relative migration times of the aminopyridines upon changes of the buffer pH, buffer molarity, applied voltage, and detection wavelength compared to standard assay conditions[a]

Compound	Standard conditions[b]	Buffer pH		Buffer molarity		Applied voltage		Detection wavelength	
		pH 5.10	pH 5.20	90 mM	110 mM	19 kV	21 kV	238 nm	242 nm
4-AP	0.681 (0.08%)	0.674 (0.33%)	0.683 (0.14%)	0.679 (0.17%)	0.676 (0.12%)	0.669 (0.17%)	0.672 (0.13%)	0.683 (0.15%)	0.680 (0.49%)
2-AP	0.709 (0.17%)	0.702 (0.33%)	0.712 (0.16%)	0.706 (0.14%)	0.704 (0.13%)	0.697 (0.15%)	0.701 (0.13%)	0.711 (0.13%)	0.708 (0.35%)
3-AP	0.718 (0.28%)	0.711 (0.35%)	0.728 (0.12%)	0.716 (0.18%)	0.715 (0.08%)	0.707 (0.10%)	0.711 (0.11%)	0.723 (0.11%)	0.718 (0.44%)
3,4-DAP	0.733 (0.16%)	0.727 (0.28%)	0.734 (0.17%)	0.730 (0.07%)	0.728 (0.14%)	0.722 (0.12%)	0.725 (0.14%)	0.738 (0.22%)	0.732 (0.29%)
2,3-DAP	0.756 (0.15%)	0.751 (0.25%)	0.759 (0.14%)	0.753 (0.14%)	0.752 (0.10%)	0.746 (0.08%)	0.749 (0.08%)	0.760 (0.18%)	0.755 (0.38%)
2,6-DAP	0.772 (0.14%)	0.767 (0.22%)	0.777 (0.09%)	0.770 (0.08%)	0.769 (0.05%)	0.765 (0.14%)	0.765 (0.12%)	0.775 (0.13%)	0.771 (0.32%)

[a] Each sample was injected five times. RSDs are listed in brackets.
[b] Standard conditions: 100 mM sodium acetate buffer, pH 5.15; applied voltage, 20 kV; detection wavelength, 240 nm.

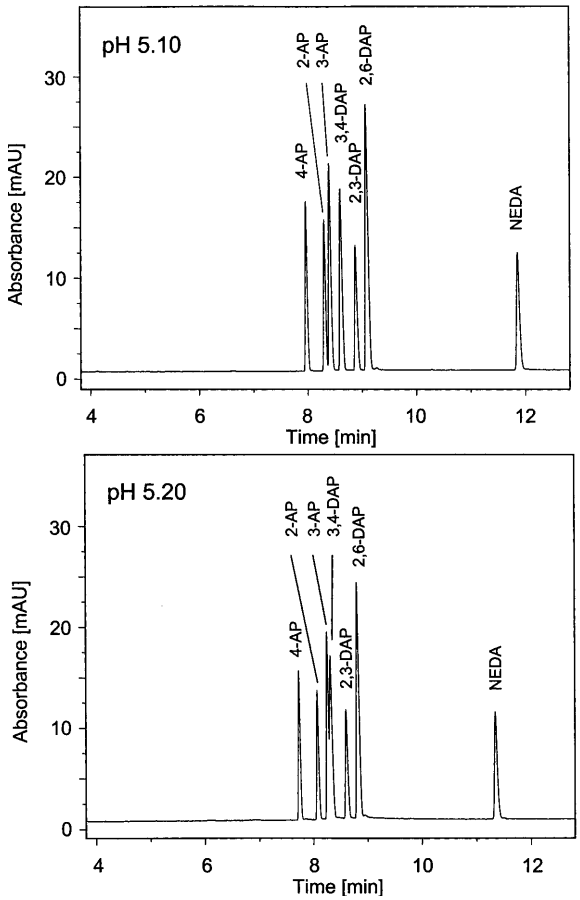

Fig. 4. Electropherogram of standards at pH 5.10 (top) and pH 5.20 (bottom). Other conditions and abbreviations see Fig. 2.

gram at pH 5.15 (Fig. 2) 3-aminopyridine migrates closer to 2-aminopyridine at pH 5.10, but the compounds are still almost baseline separated. At pH 5.20, 3-aminopyridine migrates close to 3,4-diaminopyridine with the peaks overlapping partially. At pH 5.00 and pH 5.25, 3-aminopyridine comigrates with 2-aminopyridine and 3,4-diaminopyridine, respectively (data not shown). The resolution of the other compounds was not changed significantly by small changes of the buffer pH.

The effect of the buffer pH, buffer molarity, applied voltage and detection wavelength on the corrected peak areas ratio of the isomeric aminopyridines relative to the internal standard is summarized in Table 5. Except for changes of the detection wavelength, variation of the other parameters did not significantly alter the corrected area ratios. The deviation of the area ratios relative to the area ratio under standard conditions defined as 100% never exceeded 5%. The fact that a higher RSD within the series at pH 5.20 was observed for 3-aminopyridine and 3,4-diaminopyridine is due to the partial overlap of the peaks at this pH (Fig. 4). This led to a higher variation of the peak area determined by the integration software. As expected, a large variation of the area ratios was found upon changing the detection wavelength (Table 5). None of the compounds was measured at the respective UV maximum. Thus, small changes will significantly effect the area as the large variations of the absorbance coefficients may occur within a few nm. However, changes of the wavelength within a series of measurements are not likely when using reliable instruments. Nevertheless, the analysis of reference samples is highly recommended in order to identify any variations of the detection wavelength.

4. Conclusions

A CE method for the simultaneous analysis of isomeric aminopyridines and diaminopyridines was developed and validated with regard to linearity, range, limit of quantitation and detection, precision, specificity and robustness. The assay was found to be precise, accurate and robust allowing the identification and quantitation of all compounds. As demonstrated for 3,4-diaminopyridine, the method may also be used within certain limits to test for the other pyridine derivatives as potential impurities at the 0.1% level. Thus, in agreement with other studies [11–22], CE may be a powerful tool for the determination and purity control of drug substances. The present assay was not optimized for a specific pyridine derivative. Thus, if applied to the analysis or detection of a specific aminopyridine, detection should be carried out at the UV maximum of the compound of interest in order to further increase the sensitivity of the method.

Table 5
Robustness of the corrected peak area ratios of the aminopyridines versus internal standard upon changes of the buffer pH, buffer molarity, applied voltage, and detection wavelength compared to standard assay conditions[a]

Compound	Standard conditions[b]	Buffer pH		Buffer molarity		Applied voltage		Detection wavelength	
		pH 5.10	pH 5.20	90 mM	110 mM	19 kV	21 kV	238 nm	242 nm
4-AP	1.168 (0.75%)	1.196 (1.60%)	1.224 (0.57%)	1.248 (0.69%)	1.232 (0.71%)	1.190 (0.44%)	1.197 (0.42%)	0.917 (0.76%)	1.454 (0.66%)
2-AP	1.185 (0.64%)	1.144 (1.27%)	1.164 (0.73%)	1.174 (0.60%)	1.166 (0.28%)	1.182 (0.21%)	1.184 (0.27%)	1.429 (0.40%)	0.939 (0.66%)
3-AP	1.651 (0.30%)	1.649 (1.47%)	1.569 (1.76%)	1.721 (0.77%)	1.698 (0.60%)	1.670 (0.26%)	1.678 (0.20%)	1.537 (0.29%)	1.777 (0.46%)
3,4-DAP	1.685 (0.39%)	1.633 (1.25%)	1.766 (3.04%)	1.675 (0.58%)	1.666 (0.43%)	1.679 (0.13%)	1.687 (0.24%)	2.168 (0.16%)	1.293 (0.37%)
2,3-DAP	0.967 (0.41%)	0.962 (1.31%)	0.992 (0.60%)	1.000 (0.83%)	0.997 (0.98%)	0.979 (0.47%)	0.977 (0.35%)	0.942 (0.39%)	1.011 (0.60%)
2,6-DAP	2.333 (0.29%)	2.353 (1.01%)	2.367 (0.55%)	2.412 (0.37%)	2.386 (0.48%)	2.356 (0.28%)	2.367 (0.15%)	2.203 (0.31%)	2.410 (0.38%)

[a] Each sample was injected five times. RSDs are listed in brackets.
[b] Standard conditions: 100 mM sodium acetate buffer, pH 5.15; applied voltage, 20 kV; detection wavelength, 240 nm.

Acknowledgements

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