Journal of Chromatography A, 652 (1993) 495–501
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 307

Separation of water-soluble vitamins via high-performance capillary electrophoresis

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ABSTRACT

A standard sample and method for performance evaluation in capillary zone electrophoresis is defined. The sample, containing thiamine, nicotinamide and nicotinic acid, was analysed in a 20 m*M* sodium phosphate buffer pH 7. The impact of pH, buffer type and ionic strength on electroosmotic flow, electrophoretic mobility, and peak shape was investigated. The separation of several water-soluble vitamins in phosphate buffer pH 7 was developed in order to analyse an over-the-counter vitamin preparation. Spectral analysis and peak purity tests were applied on-line for peak identification.

INTRODUCTION

Capillary electrophoresis (CE) is a relatively new analytical method currently under investigation for routine use in industrial laboratories. There is still no complete understanding of parameters that influence the separation process for CE. The purpose of this investigation was to define a standard sample and method for performance evaluation and system suitability test in capillary zone electrophoresis (CZE). It is desirable for the standard sample and method to have the following features:

(1) The standard should contain substances of different charge types to demonstrate the separation principle of the zonal technique.

(2) To provide good signal-to-noise ratios, the substances should have high molecular extinction coefficients in the UV–Vis range.

(3) The standard separation should provide good peak area and time reproducibility.

The vitamins thiamine, nicotinamide and nicotinic acid satisfy these requirements.

These well-defined standards allow the study of fundamental parameters in CZE. In this context the impact of different analysis conditions, such as buffer type, ionic strength and pH, on the performance of the standard separation were investigated. In addition, a separation method for several water-soluble vitamins was developed in order to analyse an over-the-counter vitamin tablet preparation. This study demonstrates the impact of matrix effects in CZE.

Previously, separations of vitamins have been reported using high-performance liquid chromatography (HPLC) [1–5]. This technique requires gradient elution and suffers from poor efficiency and peak tailing [4,5]. In CE, vitamins have been separated using micellar electrokinetic capillary chromatography (MECC) [6–9]. The application of micelles leads to complex equilibrium states before separation occurs. This complexity and diversity makes understanding and optimization difficult. In this study, a simplified approach using CZE was taken.

EXPERIMENTAL

Apparatus

A Hewlett-Packard (Waldbronn, Germany) three-dimensional capillary electrophoresis system was used in all measurements. The fused-

0021-9673/93/$06.00

silica capillaries (50 μm I.D., straight and with extended path length detection cell) are available from Hewlett-Packard.

Chemicals

Thiamine hydrochloride (vitamin B_1 hydrochloride), nicotinamide (vitamin PP), nicotinic acid (vitamin B_3), pyridoxine hydrochloride (vitamin B_6 hydrochloride), cyanocobalamin (vitamin B_{12}), folic acid calcium salt pentahydrate (vitamin B_8), all BioChemika grade, and orotic acid (vitamin B_{13}) of analytical grade were obtained from Fluka (Buchs, Switzerland). L-Ascorbic acid (vitamin C) and calcium D (+) pantothenate, both biochemistry grade, were obtained from Merck (Darmstadt, Germany). HPCE-grade sodium phosphate buffer pH 7, sodium tetraborate buffer pH 8 and sodium citrate buffer pH 5, all 20 m*M*, were provided by Fluka.

Sample preparation

The standard vitamins, thiamine, nicotinamide and nicotinic acid, were dissolved in water (each 0.001 *M*). The resulting pH of the solution was 3.5. The samples including all vitamin compounds (each 0.001 *M*, except calcium folinate and ascorbic acid, each 0.015 *M*) were dissolved in citrate buffer pH 5 since folinate is not stable at lower pH values. The over-the-counter vitamin preparation was ground, dissolved in pH 5 buffer, filtered through a 0.2-μm syringe filter and injected immediately.

Analysis conditions

For optimal performance, the capillaries were preconditioned for 10 min with 1.0 *M* NaOH, for 2 min with 0.1 *M* NaOH and finally for 3 min with run buffer before the first use. Between runs, capillaries were flushed for 2 min with 0.1 *M* NaOH and 3 min with run buffer. In order to ensure area reproducibility, subsequent injection of a buffer plug was necessary.

RESULTS AND DISCUSSION

Standard separation method

The vitamins thiamine, nicotinamide and nicotinic acid selected as standards were separated in a fused-silica capillary. Sodium phosphate buffer pH 7, 20 m*M*, was used as electrolyte. Several electropherograms of a sequence of 42 runs are shown in Fig. 1.

Through the relationship of mobility vectors:

$$\mu_{total} = \mu_{EOF} + \mu_{mobility} \quad (1)$$

it is anticipated that, given a constant electroosmotic flow (EOF), components will separate reproducibly according to their electrophoretic mobility. In this example the first-eluting compound is expected to be thiamine, which carries a permanent positive charge. Since nicotinamide is neutral at pH 7, it is transported with the velocity of the EOF and can therefore be used as an EOF marker. Nicotinic acid, which is negatively charged at the chosen pH, eluted last.

The average migration times and areas as well as the values for the relative standard deviation (R.S.D.) are given in Table I. The R.S.D. values of all compounds were about 0.5% for migration time and 1.7% for the peak area over 42 injections. Theoretical plate numbers of 179 809,

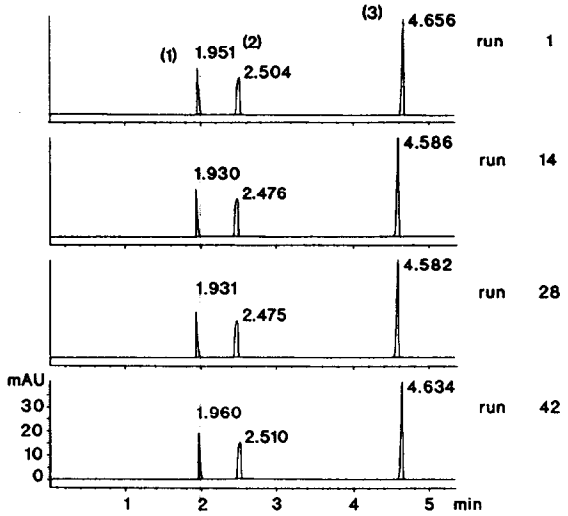

Fig. 1. Reproducibility of the standard separation method. Capillary: fused-silica, I.D. 50 μm, straight, length to detector 400 mm, total length 485 mm. Sample and peak identification: 1 = thiamine hydrochloride; 2 = nicotinamide; 3 = nicotinic acid dissolved in water. Injection pressure: 4.6 s at 40 mbar. Post-injection pressure: 4 s at 40 mbar. Run buffer: 20 m*M* sodium phosphate pH 7, run buffer replenishment every third run. Separation: polarity positive, voltage 20 kV, capillary temperature 25°C. Detection: 215 nm.

TABLE I

REPRODUCIBILITY OF STANDARD VITAMIN SEPARATION

Capillary: fused-silica, I.D. 50 μm, straight, length to detector 400 mm, total length 485 mm. Sample: 0.001 *M* each thiamine hydrochloride, nicotinamide, nicotinic acid (in water). Injection pressure: 4.6 s at 40 mbar. Post-injection pressure (run buffer): 4 s at 40 mbar. Run buffer: 20 m*M* sodium phosphate buffer pH 7, run buffer replenishment every third run. Separation: polarity positive, voltage 20 kV, capillary temperature 25°C.

	Migration time (min)	Peak width	Peak area (not time corrected)
Thiamine			
Average (42 runs)	1.937	0.017	25.38
R.S.D. (%)	0.52	1.58	1.68
Nicotinamide			
Average	2.485	0.048	43.40
R.S.D. (%)	0.49	8.36	1.67
Nicotinic acid			
Average	4.600	0.025	69.04
R.S.D. (%)	0.48	0.61	1.71

371 121 and 468 906 per metre were calculated for the peaks of thiamine, nicotinamide and nicotinic acid, respectively.

Influencing parameters

The impact of pH, buffer type and ionic strength on EOF and electrophoretic mobility of the solutes as well as on the peak shape was examined.

Four different buffers —sodium phosphate buffer pH 7 and 8, sodium tetraborate buffer pH 8 and sodium citrate buffer pH 6— were used to test the influence of the described parameters. The concentration of each buffering ion was 20 m*M*. The ionic strength of these buffers was increased from tetraborate pH 8 to phosphate pH 7 and pH 8 and to citrate pH 6 using sodium as the counter ion. The data for ionic strength of the buffers, the EOF and the electrophoretic mobilities of the solutes are summarized in Table II. Increasing the pH of buffers used in unmodified fused-silica capillaries is known to in-

TABLE II

IONIC STRENGTH, ELECTROOSMOTIC FLOW AND ELECTROPHORETIC MOBILITIES CALCULATED FOR DIFFERENT BUFFERS

Capillary: fused-silica, I.D. 50 μm, length to detector 608 mm. Sample: 0.001 *M* each thiamine hydrochloride, nicotinamide, nictonic acid (in water). Injection pressure: 4.6 s at 40 mbar. Post-injection pressure: 4 s at 40 mbar. Run buffer 1: 20 m*M* sodium phosphate buffer pH 7. Run buffer 2: 20 m*M* sodium phosphate buffer pH 8. Run buffer 3: 20 m*M* sodium tetraborate buffer pH 8. Separation: polarity positive, voltage 25 kV, capillary temperature 25°C.

Buffer	Ionic strength	Thiamine mobility (10^{-4} cm^2 V^{-1} s^{-1})	EOF (10^{-4} cm^2 V^{-1} s^{-1})	Nicotinic acid mobility (10^{-4} cm^2 V^{-1} s^{-1})
Sodium tetraborate pH 8	0.0235	0.93	5.63	−1.94
Sodium phosphate pH 7	0.0352	1.04	3.91	−1.77
Sodium phosphate pH 8	0.0548	1.03	3.77	−1.78
Sodium citrate pH 6	0.0772	1.45	5.54	−2.90

crease the EOF. In contrast, increasing the ionic strength has the opposite effect: the EOF is decreased. In the series of tetraborate buffer to phosphate buffers of pH 7 and 8 listed in Table II the EOF decreased as expected. The electrophoretic mobilities of thiamine and nicotinic acid changed only little in this series. Although in the case of citrate buffer both the pH and the ionic strength led to the expectation that the EOF should decrease further, the opposite occurred. The electrophoretic mobilities of the solutes in citrate buffer differed significantly compared with those observed in the other three buffers. This effect is not yet understood. Further investigations are under way. Comparison of the EOF measured for the different buffers and the electrophoretic mobilities of the vitamin compounds in these buffers (Table II) indicated that, besides the ionic strength and the pH, the buffer type is a third important parameter determining the EOF and the zeta potential. Depending on the buffer type, different kinds of interactions of buffering ions with the silica surface occur. Phosphate is known to chemically modify the fused-silica surface [10,11], whereas other anions dynamically interact with the surface. Thus buffer ion type may have an influence not only on the surface zeta potential and on the counter-ion shielding of the solute, but also on the nature of solute–surface interactions. Systematic measurements with more buffer systems are necessary to clarify these assumptions.

The ionic strength of a run buffer has an important impact on the peak shape and therefore on the efficiency of the separation. Fig. 2 shows a series of standard vitamin separations in 20 m*M* tetraborate buffer solution pH 8, in which the ionic strength has been increased by adding the neutral salt sodium chloride to the run buffer. The amounts of added salt are summarized in Table III. This table also shows the number of theoretical plates calculated for the three standard vitamins depending on the ionic strength of the buffer system. With increasing sodium concentration the plate numbers of the thiamine peaks increased strongly and the tailing peak developed to an almost Gaussian peak. This behaviour can be explained by competitive ion-exchange properties of sodium ions

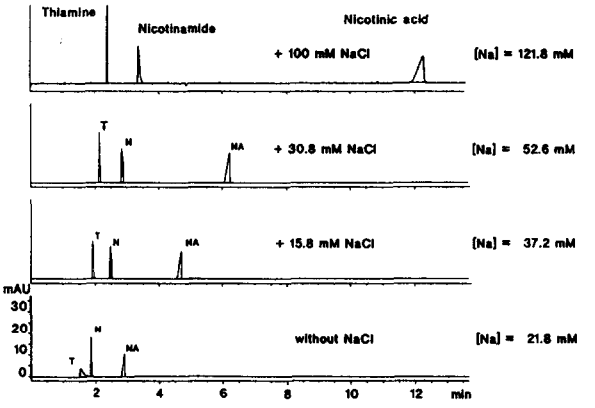

Fig. 2. Peak shape dependence on ionic strength. The ionic strength of a 20 m*M* sodium tetraborate buffer pH 8 was increased by adding different amounts of the neutral salt sodium chloride to the solution. For conditions, see Fig. 1.

versus the positively charged thiamine. In contrast, the peak shape of nicotinic acid is determined by isotachophoretic effects. The fronting effect of the peak increased with increasing conductivity of the anions in the buffer (Fig. 2). It has to be considered that within the test set-up not only the conductivity of the buffer was changed but also the electrolyte type from mainly tetraborate to mainly chloride. It is known from the theory of isotachophoretic separations that electrolyte type has an impact on solute mobility.

Separation of water-soluble vitamins

The separation of the eight water-soluble vitamins was performed under the sample conditions as previously stated except that a longer capillary with an extended path length detection cell was used. The capillary was selected to improve separation and detection properties. Fig. 3 shows the separation of the vitamins at different wavelengths optimized for the different analytes. Peak identification was performed by single analyte injection and on-line spectral comparison. Peak purity was tested by measurements of spectral differences at several points of the peak considered. These tests indicated no impurities in the obtained peaks.

Using buffer without micellar additives for the analysis an electrophoretic separation of neutral vitamins is not possible. Nevertheless, a quantitative determination of these compounds is

TABLE III

NUMBER OF THEORETICAL PLATES DEPENDING ON IONIC STRENGTH

Capillary: fused-silica, I.D. 50 μm, straight, length to detector 400 mm, total length 485 mm. Sample: 0.001 *M* each thiaminehydrochloride, nicotinamide, nicotinic acid (in water). Injection pressure: 4.6 s at 40 mbar. Post-injection pressure (run buffer): 4 s at 40 mbar. Run buffer: 20 m*M* sodium tetraborate pH 8 plus various amounts of sodium chloride, run buffer replenishment. Separation: polarity positive, voltage 20 kV, capillary temperature 25°C. *N* = Number of theoretical plates.

	N/m			
	Tetraborate without NaCl	Tetraborate +15.4 m*M* NaCl	Tetraborate +30.8 m*M* NaCl	Tetraborate +100 m*M* NaCl
Thiamine	5120	74 900	135 993	273 975
Nicotinamide	15 111	36 823	36 123	62 843
Nicotinic acid	66 762	101 139	109 301	85 515

possible by using spectral suppression. This is shown for nicotinamide and cyanocobalamin in the electropherograms of Fig. 4, in which both vitamins are separated from other vitamins. The determination of nicotinamide was performed at 260 nm, at which wavelength the spectral absorbance of cyanocobalamin could be suppressed by its reference absorbance at 285 nm. Cyanocobalamin has an absorption maximum at 360 nm and a lower one at 550 nm. As nicotinamide showed no absorption at these wavelengths, a quantitative determination of cyanocobalamin can be carried out without any interference.

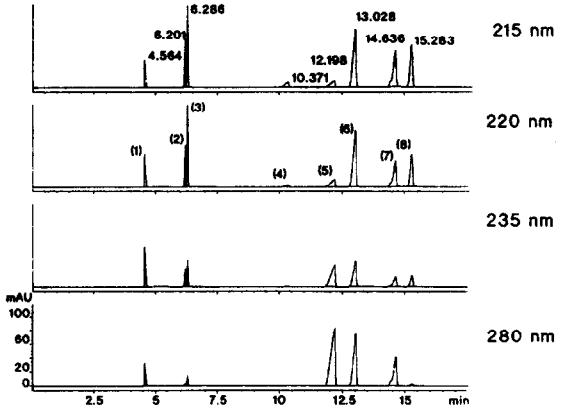

Fig. 3. Separation of eight water-soluble vitamins. Capillary: fused-silica, I.D. 50 μm, extended path length detection cell, length to detector 560 mm, total length 645 mm. Sample and peak identification: 1 = thiamine hydrochloride; 2 = nicotinamide; 3 = pyridoxine; 4 = pantothenate; 5 = ascorbic acid; 6 = folinate; 7 = orotic acid; 8 = nicotinic acid dissolved in citrate buffer pH 5. For conditions, see Fig. 1.

Analysis of an over-the-counter vitamin preparation

The above-described separation of eight water-soluble vitamins was taken as reference for determining the ingredients of an over-the-counter vitamin preparation (tablet). Fig. 5 shows the analysis of the vitamin tablet in comparison with the reference separation of the vitamins in phosphate buffer pH 7. Thiamine, nicotinamide, pyridoxine, pantethonate and ascorbic acid were identified. The identification was only possible

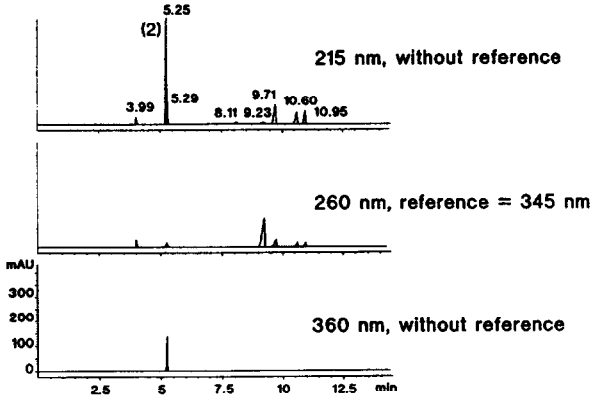

Fig. 4. Analysis of nine water-soluble vitamins using spectral suppression. For sample and peak identification, see Fig. 3, except that here peak 2 = cyanocobalamin. For conditions, see Fig. 3.

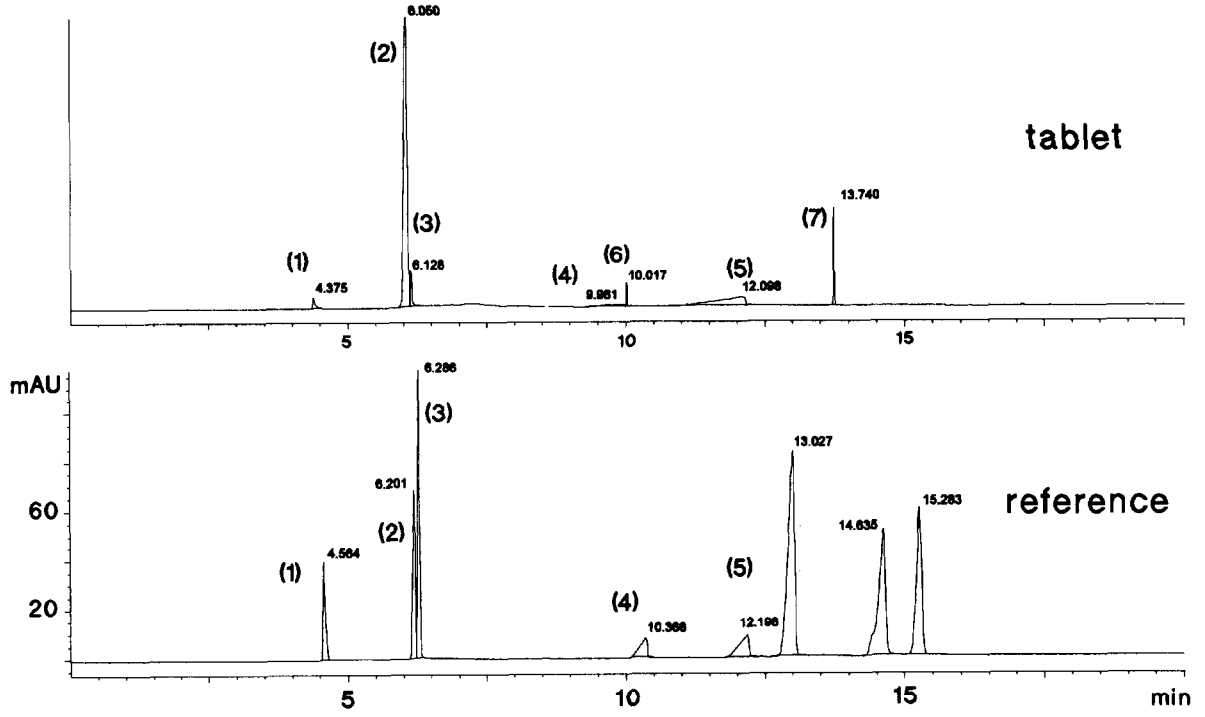

Fig. 5. Comparison of tablet analysis with the reference sample containing eight vitamins. Peak identification: 1 = thiamine; 2 = nicotinamide; 3 = pyridoxide; 4 = pantothenate; 5 = ascorbic acid; 6 and 7 = matrix peaks. For conditions, see Fig. 3.

by measuring and comparing spectra because the interfering matrix influenced migration times and peak shapes. The spectra of peaks 6 and 7 (Fig. 5, tablet) were unlike any vitamin spectra measured before and thus these peaks were classified as matrix peaks.

CONCLUSIONS

The vitamins thiamine, nicotinamide and nicotinic acid turned out to be well suited for a standard sample set in CZE. Excellent repeatability and performance of the standard method could be shown.

Of the tested parameters, pH had a minor impact on the standard separation procedure over the range studied. Parameters such as ionic strength and buffer type strongly influenced the EOF, electrophoretic mobility of the solutes and peak shapes. When analysing an over-the-counter vitamin preparation, shifts in migration times and peak shapes were observed. This led to the conclusion that complex matrices influence these parameters.

The determination of nine compounds and the analysis of a commercially available vitamin preparation showed that vitamin analysis is possible with a simple buffer system, avoiding difficult micellar techniques. Spectral analysis and peak purity tests proved to be useful for peak identification.

ACKNOWLEDGEMENTS

The author wishes to thank Sally Swedberg for helpful discussions, Petra Seitz and Gerard Rozing for comments on the manuscript and the Capillary Electrophoresis Group for support in daily work.

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