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Determination of six water-soluble vitamins in a pharmaceutical formulation by capillary electrophoresis

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Abstract

A method was developed for the quantitative analysis of six water-soluble vitamins (thiamine, nicotinamide, riboflavine, pyridoxine, ascorbic acid and pantothenic acid) in a pharmaceutical formulation, using free solution capillary zone electrophoresis (CZE) in uncoated fused silica capillaries and UV detection. The influence of different parameters, such as the nature of the buffer anionic component and buffer concentration on the CZE separation of vitamins was investigated using four vitamins of the B group as model compounds. A good compromise between resolution, analysis time and analyte stability was obtained by use of a 50 mM borax buffer of pH 8.5. This CZE method was found to be very useful for the separation of more complex samples, a mixture of ten water-soluble vitamins being completely resolved in about 10 min. However, cyanocobalamine could not be separated from nicotinamide in this CZE system, the two compounds being in uncharged form at the pH used. These two compounds could easily be resolved by micellar electrokinetic chromatography (MEKC), the anionic surfactant dodecylsulfate being added to the running buffer at 25 mM concentration. In the pharmaceutical formulation, some excipients were found to be adsorbed to the capillary surface, giving rise to a progressive decrease of the electroosmotic flow and consequently to a simultaneous increase of analyte migration times. A capillary wash with sodium hydroxide had to be made between successive runs in order to minimize these effects. Good results with respect to linearity, precision and accuracy were obtained in the concentration range studied for the six vitamins, using nicotinic acid as internal standard. © 1997 Elsevier Science B.V.

Keywords: Capillary electrophoresis; Water-soluble vitamins; Pharmaceutical formulation; Method development; Method validation

1. Introduction

Multi-vitamin preparations are widely used in therapeutics to supply for the possible lack of these nutrients, essential for the functioning of the human organism. The vitamins of the B group, which are the most important constituents of water-soluble vitamins, have very different chemical structures. Water-soluble vitamins are available in many pharmaceutical formulations, most of them containing various kinds of excipients. Consequently, the quality control of these complex preparations needs very powerful separation techniques. So far, the simultaneous determination of a limited number of water-soluble vitamins could be performed by high performance liquid

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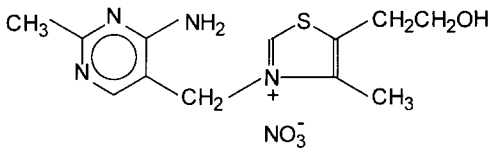

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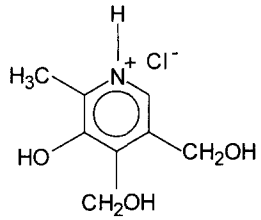

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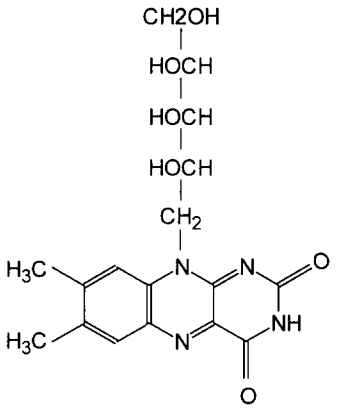

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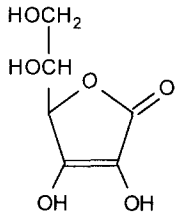

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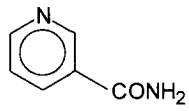

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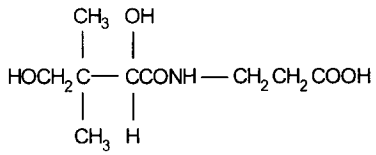

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Fig. 1. Structures of the six water-soluble vitamins examined. A, thiamine mononitrate; B, riboflavine; C, nicotinamide; D, pyridoxine hydrochloride; E, ascorbic acid; F, pantothenic acid.

chromatography (HPLC). Due to its high efficiency, capillary electrophoresis (CE) appears as an appropriate technique for the analysis of this type of formulation, as demonstrated in several papers published in this field [1–8]. Micellar electrokinetic chromatography (MEKC) was used to separate mixtures of vitamins of the B group (thiamine, riboflavine, nicotinamide and pyridoxine) [1–5] containing possibly also cyanocobalamine [1–4], ascorbic acid [1–4] and calcium pantothenate [4]. Sodium dodecylsulfate (SDS) was most often used as anionic surfactant, at concentrations from 22.5 mM [4] to 50 mM [1–3] or 100 mM [5], in buffers in pH range 7–9. A comparison between the use of SDS and sodium lauroylmethyl taurate (LMT) as surfactant was also reported [2]. More recently, methods for the determination of the same water-soluble vitamins by capillary zone electrophoresis were described [5–8], using pH 9 borate [5] or pH 7–9 phosphate buffers [6–8]. Under these conditions, nicotinamide and cyanocobalamine are in uncharged form and coelute at the time corresponding to the electroosmotic flow. However, these two compounds could be quantified by CZE without interference by using two different wavelengths [6].

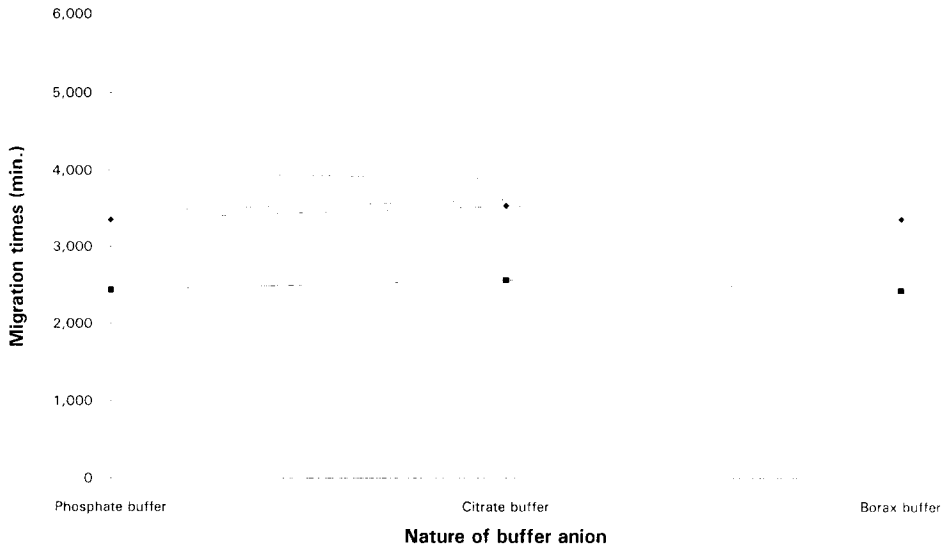

Fig. 2. Nature of buffer components on migration times. Buffer: 50 mM buffer of pH 8.5. Other conditions as described in Section 2. Solutes: ■ thiamine, □ riboflavine, ◆ nictinamide, ◇ pyridoxine.

Water-soluble vitamins were determined in different kinds of pharmaceutical formulations, such as tablets [3–6,8], injections [1], syrup and gelatine capsules [5], using MEKC [1,3–5] or CZE [6–8] methods. Ascorbic acid was also determined by CZE in fruits [7] or fruit juices [9], in the presence of L-cysteine [7] or DL-homocysteine [9] as an antioxidant.

In the case of tablets or gelatine capsules, peak deformation [4–6] and increasing migration times [5,6] have been observed, probably due to interferences from the excipients. For gelatine capsules, this problem could be solved by using MEKC, the SDS micelles dissolving probably the oily droplets present in this preparation [5].

In this paper, a fast CZE method for the simultaneous determination of six water-soluble vitamins (thiamine, riboflavine, nicotinamide, pyridoxine, ascorbic acid and pantothenic acid, Fig. 1) in tablets has been developed and validated. The possibilities of applying this method to the separation of other water-soluble vitamins has also been investigated, as well as the influence of the addition of the anionic surfactant dodecylsulfate to the running buffer.

2. Materials and methods

2.1. Apparatus

All experiments were performed using a Model ^{3D}CE system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array detector, an automatic injector, an autosampler and a temperature control system (15–60 ± 0.1°C). An HP Vectra 486/66XM computer was used for instrument control and data handling. The pH of the buffers was adjusted by means of a Delta 345 pH meter (Mettler, Healstead, UK).

2.2. Chemicals

Sodium tetraborate was obtained from Sigma (St. Louis, MO, USA). Boric acid, trisodium citrate, sodium dihydrogen phosphate and sodium dodecylsulphate of analytical grade were purchased from Merck (Darmstadt, Germany). Water was of Milli-Q quality (Millipore, bedford, MA, USA). Thiamine mononitrate (vitamin B1), riboflavine (vitamin B2), nicotinamide (vitamin B3 or PP), pyridoxine hydrochloride (vitamin B6), ascorbic acid (vitamin C), calcium pantothenate (vitamin B5) and biotin (vitamin H) were pro-

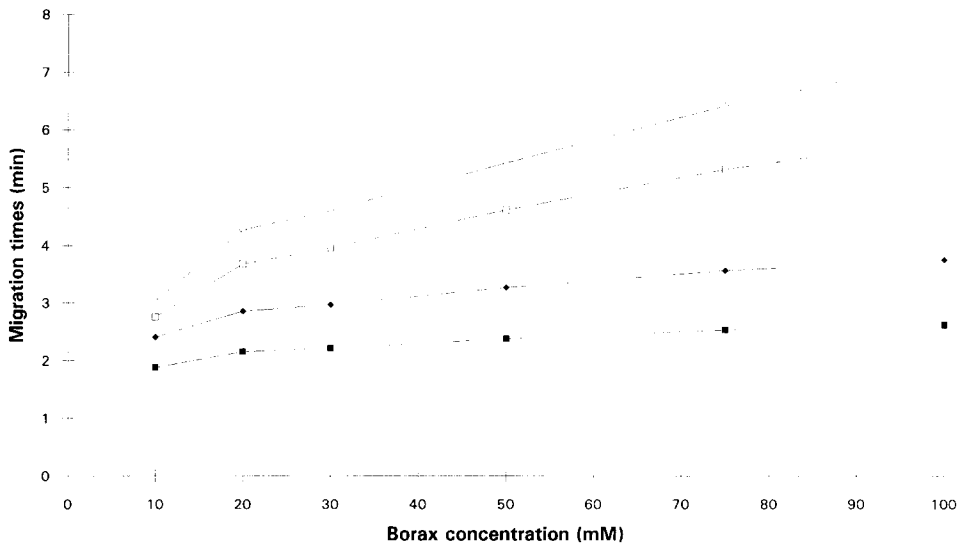

Fig. 3. Influence of buffer concentration on migration times. Buffer: 10–100 mM borate adjusted to pH 8.5 with boric acid. Other conditions as described in Section 2. Solutes: ■ thiamine, □ riboflavine, ◆ nicotinamide, ◇ pyridoxine.

vided by SMB Technology (Marche-en-Famenne, Belgium). Cyanocobalamine (vitamin B12), nicotinic acid, rutin (vitamin P) and adenine (vitamin B4) were from Sigma (St. Louis, MO, USA). All solutions were filtered through a 0.22 μm cellulose acetate membrane (Sartorius, Göttingen, Germany) before use.

2.3. Electrophoretic method

Electrophoretic separations were carried out with uncoated fused silica capillaries having 50 μm internal diameter and 48.5 cm length (40 cm to the detector) (Hewlett-Packard, Palo Alto, CA, USA). Before use, the capillary was washed successively with basic solutions (i.e., 1 M NaOH followed by 0.1 M NaOH), water and running buffer. At the beginning of each working day, the capillary was washed with separation buffer for 10 min. The latter consisted of 0.05 M sodium tetraborate (borax) adjusted to pH 8.5 with boric acid, 0.05 M trisodium citrate adjusted to pH 8.5 with phosphoric acid or 0.05 M sodium dihydrogen phosphate adjusted to pH 8.5 with sodium hydroxide. After each injection, the capillary was washed with 0.1 M sodium hydroxide for 7 min, water for 1 min and running buffer for 3 min.

The applied voltage was 25 kV and UV detection was performed at 225 nm for thiamine, riboflavine, nicotinamide and ascorbic acid, and at 215 nm for pyridoxine and pantothenic acid. Injections were made using the hydrodynamic mode (injection pressure 5 kPa) for 10 s. The capillary was thermostatted at 25°C.

2.4. Sample solutions

Using the same proportions of the six water soluble-vitamins as those in the pharmaceutical formulation investigated, standard solutions were first prepared for each calibration level ($n = 5$) by dissolving all vitamins in a 100 ml volumetric flask protected from light and containing 50 ml of a 1% acetic acid solution as dissolution medium. After heating at 65°C and simultaneous shaking for 10 min, a 5 ml volume of the internal standard solution was added the volumetric flask. The latter was filled to the mark with the dissolution medium after cooling at room temperature. The stock solution of internal standard was prepared by dissolving 500 mg of nicotinic acid in 50 ml of the same dissolution medium. All aqueous standard solutions were then filtered through a 0.22 μm cellulose acetate membrane. Before subse-

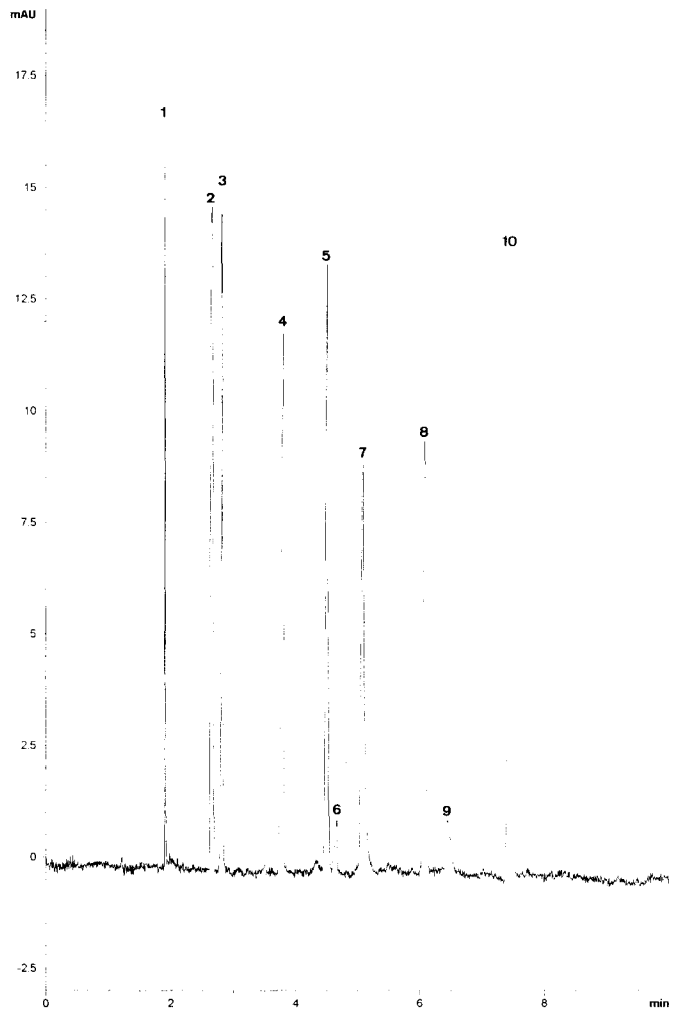

Fig. 4. CZE separation of ten water soluble vitamins. Buffer: 50 mM borax adjusted to pH 8.5 with boric acid. Detection wavelength: 225nm. Other conditions as described in Section 2. Peaks: (1) thiamine; (2) nicotinamide; (3) adenine; (4) riboflavine; (5) pyridoxine; (6) biotin; (7) rutin; (8) ascorbic acid; (9) panthotenic acid; and (10) nicotinic acid.

quent dilution (from 4–12 times) according to the desired concentration level in the calibration range (50–150% of the nominal concentration), the resulting standard solutions contained 10.80 mg ml^{-1} of ascorbic acid, 3.24 mg ml^{-1} of nicotinamide, 1.08 mg ml^{-1} of pantothenic acid, 0.36 mg ml^{-1} of pyridoxine, 0.29 mg ml^{-1} of riboflavine and 0.25 mg ml^{-1} of thiamine. The migration order of the vitamins was determined by injecting diluted solutions of each vitamin (six times lower concentrations as those than mentioned above) and by spectral comparison.

The same handling procedure was then applied to five synthetic mixtures of the dosage form excipients to which equivalent amounts of each vitamin were added (spiked placebos) in order to search for possible interferences from the excipients over the calibration range tested.

The analytical procedure was finally applied to the commercial formulation containing of ascorbic acid (180.0 mg), nicotinamide (54.0 ng), calcium pantothenate (19.5 mg), pyridoxine hydrochloride (7.2 mg), riboflavine (4.8 mg), thiamine mononitrate (5.4 mg), folic acid (0.4 mg),

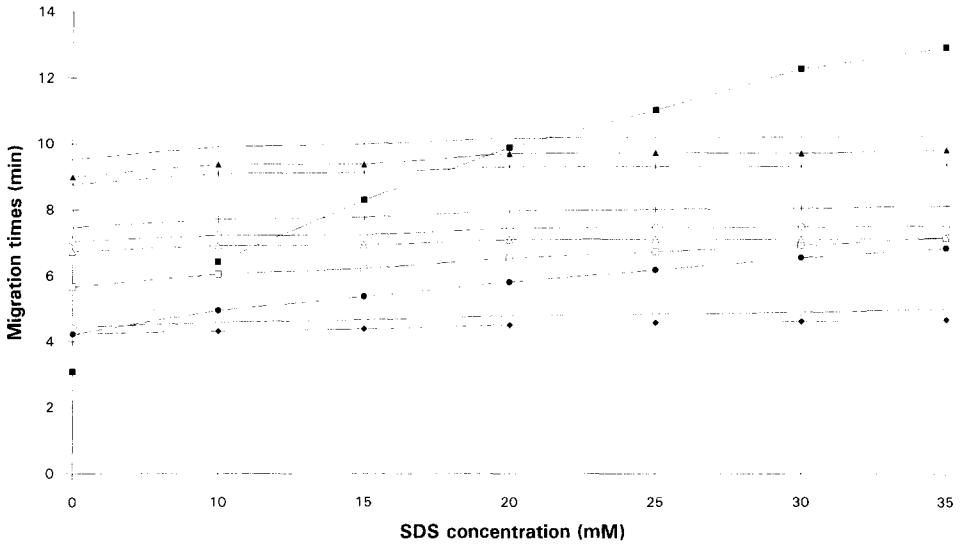

Fig. 5. Influence of SDS concentration of the separation of 11 water-soluble vitamins. Buffer: 50 mM borax adjusted to pH 8.5 with boric acid, containing 0–35 mM SDS. Detection wavelength: 225 nm. Other conditions as described in Section 2. Solutes: ■ thiamine, □ riboflavine, ◆ nicotinamide, ◇adenine, ▲ panthotenic acid, △ pyridoxine, ○ cyanocobalamine, ○ biotin, x nicotinic acid, * ascorbic acid, + rutin.

biotin (0.045 mg), alpha tocopherol acetate (30.0 mg), cellulose, silica dioxide, magnesium stearate, stearic acid, povidone, starch, lactose, methylhydroxypropylcellulose, polysorbate 60, paraffine, sodium dodecylsulfate and opaspray solution (mixture of titane dioxide, hydroxypropylcellulose, yellow orange in water) as ingredients for a tablet mass of about 800 mg. In this case, before applying the same handling procedure as described above, ten tablets were ground to an homogenous powder and a quantity of powder corresponding to one tablet was introduced in a 100 ml volumetric flask.

3. Results and discussion

3.1. Nature of buffer components

Several pH values were tested (in the range from 3.0–9.5). A buffer of pH 8.5 was selected as it provided a good stability of the vitamins and an adequate reproducibility of the migration times. Furthermore, at this pH, the high electroosmotic flow allowed for the simultaneous analysis of anionic, neutral and cationic vitamins.

As shown in Fig. 2, the best separation for the four vitamins tested as model compounds was obtained with a borax buffer in comparison to a phosphate or a citrate buffer of the same pH (8.5). The increased migration time of riboflavine could be explained by the complexation between the borate ions and the gem diols of the ribose moiety in riboflavin, giving rise to an increased dissociation of these groups and therefore a higher negative charge for this vitamin [5]. A similar type of interaction might be responsible for the higher migration time of pyridoxine in the presence of borate ions, which would also result in an increase of the negative charge of this compound. These effects led to a larger migration range and consequently to much higher resolution values between the four vitamins with the borax buffer (Fig. 2).

The electroosmotic flow was approximately the same in the presence of the three buffers tested, as indicated by the fairly constant migration time of nicotinamide, which is in uncharged form under

Table 1
Influence of NaOH washing on repeatability (RSD%) of migration times ($n = 10$)

Washing sequence (min)	Thiamine	Nicotinamide	Riboflavine	Pyridoxine	Ascorbic acid	Pantothenic acid
Water (4) buffer(3)	3.8	5.1	7.4	9.6	17.0	17.0
NaOH 0.1 M (3) water (1) buffer (3)	1.1	1.4	1.7	2.1	3.0	3.1
NaOH 0.1 M (5) water (1) buffer (3)	0.94	1.1	1.2	1.5	2.3	2.2
NaOH 0.1 M (7) water (1) buffer (3)	0.23	0.24	0.31	0.43	0.68	0.53

Buffer: 50 mM borax adjusted to pH 8.5 with boric acid. Other conditions as described in Section 2.

these conditions and therefore migrates with the electroosmotic flow.

The borax buffer also gave rise to a lower current (60 μA) than the two other buffers, which was favourable with respect to peak efficiency.

3.2. Influence of buffer concentration

An increase in migration times and resolution was obtained when the concentration of the buffer was increased (Fig. 3). This effect is certainly related to the reduction of the electroosmotic flow, due to the decrease of the zeta potential at the capillary wall–solution interface.

A complete separation was already obtained at a borax concentration of 10 mM, but the choice of the buffer concentration was in fact a compromise between resolution, efficiency, peak symmetry and analysis time. An increase of buffer concentration improved the peak shape by reducing the peak deformation due to overloading effects and also led to a more important stacking effect. However, at higher buffer concentrations, the heat production by Joule effect tends to increase, giving rise to a loss of efficiency. A borax concentration of 50 mM was found to the best compromise with respect to these different parameters.

As shown in Fig. 4, the selected buffer (50 mM borax of pH 8.5) was particularly suited for the separation of water-soluble vitamins as it could lead to a complete separation of ten of them, including adenine, biotin, rutin, ascorbic acid, pantothenic acid and nicotinic acid besides the four vitamins used as models. Under these conditions, most vitamins (peaks 3 to 10) were negatively charged and migrated slower than the electroosmotic flow, except thiamine, positively charged and migrating faster than the electroosmotic flow (peak 1) and nicotinamide, uncharged and migrating with the electroosmotic flow at the electroosmotic breakthrough time (peak 2).

3.3. Influence of SDS

In the CZE system selected, cyanocobalamine and nicotinamide were both in uncharged form and therefore were not resolved. The separation of these two vitamins could be achieved by addition of SDS to the buffer (micellar electrokinetic chromatography or MEKC). Fig. 5 shows the influence of SDS concentration on the migration times of 11 water-soluble vitamins, including cyanocobalamine and nicotinamide. These two vitamins were separated due to their different degree of distribution to the micelles. The migration time of thiamine was strongly increased with the SDS concentration, most probably because of the formation of ion pairs between this positively charged vitamin and the anionic surfactant. The best separation of the 11 tested vitamins was obtained at a SDS concentration of 25 mM.

3.4. Determination of six water-soluble vitamins in a pharmaceutical formulation

The pharmaceutical formulation examined contained a series of excipients, including cellulose derivatives. During successive runs, these compounds were probably be adsorbed to the capil-

Table 2
Precision of peak areas (RSD%)

		Thiamine	Nicotinamide	Riboflavine	Pyridoxine	Ascorbic acid	Pantothenic acid
Repeatability (day = 3; n = 6)	IS—no PN	6.6	6.0	8.5	4.4	2.9	6.6
	PN—no IS	6.2	5.9	7.6	6.3	6.8	6.0
	IS and PN	1.3	1.7	2.4	1.1	1.2	2.9
Intermediate precision (day = 3; n = 6)	IS—no PN	13.0	9.1	8.5	6.8	4.6	6.6
	PN—no IS	10.0	10.0	11.9	12.4	10.3	15.4
	IS and PN	2.9	2.8	2.9	2.0	2.9	3.3

Buffer: 50 mM borax adjusted to pH 8.5 with boric acid. Other conditions as described in Section 2. PN: peak area normalisation; IS: internal standard (nicotinic acid).

lary surface. This led to a progressive decrease of the electroosmotic flow, which resulted in a concomitant increase of the migration times of the vitamins and consequently in a very low repeatability of these migration times. Cellulose derivatives are often added in CZE to suppress the electroosmotic flow [10] and are difficult to wash off the capillary. The last migrating vitamins were more influenced by this phenomenon, as demonstrated by their higher RSD values, given in Table 1. Several washing sequences for the capillary reconditioning between successive injections, involving 0.1 M NaOH, water and running buffer with different rinse times, were tested in order to remove the adsorbed excipients from the capillary wall and improve the repeatability of migration times. As shown in Table 1, acceptable RSD values, lower than 0.7%, were obtained with a washing sequence comprising rinses with 0.1 M NaOH for 7 min, water for 1 min and running buffer for 3 min. Another consequence of these adsorption phenomena is that standard solutions used in the routine analysis could preferably contain the excipients of the pharmaceutical formulation besides the active ingredients in order to match solution viscosities.

The precision of peak areas was also assessed before starting the validation process. As can be seen in Table 2, the effects of the correction of the peak area by the corresponding migration time (peak area normalisation) and of the use of an internal standard (nicotinic acid) were studied. Table 2 shows that these two techniques had a rather limited effect on the precision of the method when they were applied separately. This is especially true for peak area normalisation, the RSD values being about 6% for repeatability and generally higher than 10% for intermediate precision in this case. However, the method precision could be greatly improved by the simultaneous use of the two techniques. Under these conditions, good results were obtained for repeatability (RSD values ranging from 1.3–2.9%) and especially for the time-intermediate precision (RSD values ranging from 2.0–3.3%).

3.5. Method validation

3.5.1. Selectivity

As can be seen from Fig. 6, no interferences from the formulation excipients could observed at the migration times of the six vitamins to be determined. The small disturbance given by the blank (synthetic mixture of excipients) at the electroosmotic breakthrough time could be considered as negligible in comparison to the very large peak area given by the nicotinamide peak a the same migration time. In addition, the peak homogeneity was confirmed for each vitamin by use of a diode-array detector. For each compound, the

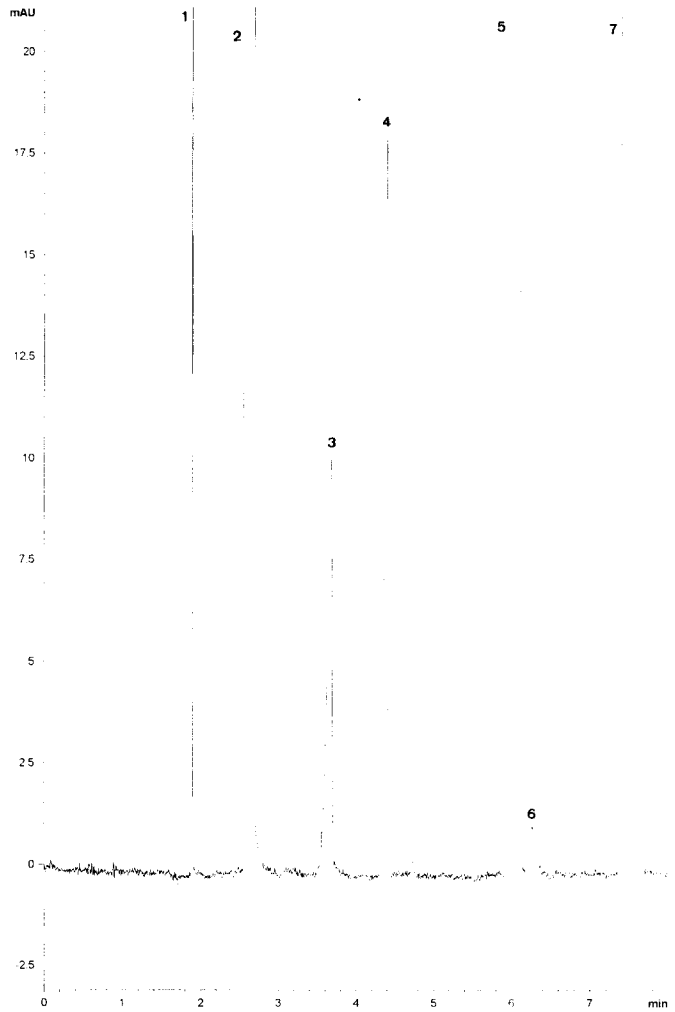

Fig. 6. CZE separation of six vitamins in a pharmaceutical formulation. Buffer: 50 mM borax adjusted to pH 8.5 with boric acid. Detection wavelength: 225 nm. Other conditions as described in Section 2 methods. Peaks: (1) thiamine; (2) nicotinamide; (3) riboflavine; (4) pyridoxine; (5) ascorbic acid; (6) panthotenic acid; and (7) nicotinic acid (internal standard).

comparison of various spectra recorded during the passage of the corresponding peak led to match scores very close (greater than 990) to the maximum values [11].

3.5.2. Linearity

For each vitamin, calibration graphs were constructed at five concentration levels in the range from 50–150% of the nominal concentration levels and three independent determinations were performed at each concentration ($n = 3$). Linear regression lines were obtained by plotting the ratios of normalised peak areas to those of the internal standard (y, arbitrary units) versus the analyte concentration (x, μg ml^{-1}) using the least squares method. The linearity of the calibration curve for each vitamin in water solution (standard solution) was first determined. In all cases straight lines passing through the origin and coefficients of determination (r^2) greater than 0.998 were obtained. The linearity was also confirmed by an analysis of variance (ANOVA) [12,13]. The same

Table 3
Linearity and accuracy of the method (spiked placebos)

Regression parameters	Thiamine	Nicotinamide	Riboflavine	Pyridoxine	Ascorbic acid	Pantothenic acid
Linearity						
r^2	0.9963	0.9979	0.9976	0.9989	0.9989	0.9967
Intercept (10^{-3})	1.3	−94	18.3	0.8	−60	2.9
Slope (10^{-3})	20.2	25.5	21.2	13.8	3.9	1.0
Accuracy						
Mean recovery ± CI (%)						
50%	99.5 ± 2.2	100.4 ± 0.5	99.8 ± 3.1	100.2 ± 0.9	98.5 ± 3.1	101.3 ± 3.2
100%	98.2 ± 1.9	98.9 ± 1.9	100.0 ± 2.3	99.7 ± 1.4	99.3 ± 1.2	100.9 ± 3.6
150%	99.1 ± 1.4	100.5 ± 0.5	99.3 ± 3.0	99.4 ± 1.0	100.9 ± 1.4	101.8 ± 2.6

Table 4
Precision of the method (spiked placebos)

Concentration levels (%)	Thiamine	Nicotinamide	Riboflavine	Pyridoxine	Ascorbic acid	Pantothenic acid
Repeatability ($k = 3$, $n = 6$, RSD%)						
50	4.4	2.0	2.3	2.5	1.4	3.6
100	1.3	1.7	2.4	1.1	1.2	2.9
150	2.0	1.9	3.0	1.3	1.0	3.4
Intermediate precision ($k = 3$, $n = 6$, RSD%)						
Concentration levels (%)						
50	4.5	3.3	3.0	2.5	1.7	4.1
100	2.9	2.8	2.9	2.0	2.9	3.3
150	2.0	2.9	3.0	1.8	1.5	4.0

statistical approach was then applied to a synthetic mixture of the excipients of the dosage form to which known quantities in each vitamin were added (spiked placebos) corresponding to the different concentration levels of the range tested. The regression parameters are given in Table 3. As earlier, the regression lines were obtained by using the least squares method according to the hypothesis of homoscedasticity [12,13]. All regression lines passed through the origin with a $t_{calc} < 2.16$ ($p = 0.05$). In most cases, the intercept values were lower than 1% of the response obtained at the 100% level, except for riboflavine and pantothenic acid (intercept values slightly higher than 2%), The linearity of the calibration graph for each vitamin was demonstrated by the high determination coefficients obtained for the regression lines and was confirmed by an ANOVA. The adequacy of the regression model chosen to describe the relationship between y and x (lack of fit test) was assessed with a $F_{calc} < 3.71$ ($p = 0.05$) for all regression lines. Finally, the slopes and intercepts of the graphs for standards and spiked placebos, respectively, were found to be not significantly different, with $t_{calc} < 2.05$ ($p = 0.05$) for each vitamin. According to the results of these statistical tests, it can be concluded that there is no matrix effect, even for nicotinamide, which migrates with the electroosmotic flow and a single-level calibration corresponding to the labelled amount of each vitamin could be used in routine analysis.

3.5.3. Accuracy

Method accuracy was determined by analysing a placebo (mixture of excipients) spiked with the six vitamins at three concentration levels ($n = 6$) covering the same range as that used for linearity

Table 5
Determination of six vitamins in a commercial tablet formulation

Analyte	Labelled amount (mg)	Amount found (mg)	% of the labelled amount
Thiamine	4.2	4.6	108.5
Nicotinamide	54.0	54.4	100.8
Riboflavine	4.8	4.5	93.6
Pyridoxine	6.0	6.4	106.5
Ascorbic acid	180.0	187.7	104.3
Pantothenic acid	18.0	17.9	99.6

(50, 100, 150%). Mean recoveries with 95% confidence intervals (C.I.) are given in Table 3. Good results with respect to accuracy were obtained for the six vitamins. The maximum experimental bias on each point is lower than 1.8% which is an acceptable value for an active ingredient in a pharmaceutical formulation. The method can thus be considered as accurate over the range investigated.

3.5.4. Precision

As shown in Table 4, method precision was determined by measuring repeatability and intermediate precision (between-day precision or time-different intermediate precision) for each vitamin in spiked palcebos ($n = 6$). This study was carried out during 3 days ($k = 3$) at the same three concentration levels (50, 100, 150%). The RSD values were estimated from repeatability and time-different intermediate precision variances, respectively [13,14]. Acceptable results with respect to precision (RSD less than 4%) were obtained for all vitamins studied. Somewhat higher RSD values were obtained however, for thiamine and pantothenic acid, most probably due to their much smaller peak areas at the 100% level (Fig. 6).

3.5.5. Quantitative analysis of a commercial multivitamin preparation

The vitamin content in a commercial tablet formulation was quantified by the CE method described above. The results are given in Table 5. They confirm that capillary electrophoresis can be considered as an interesting and cost effective alternative to liquid chromatography for the quality control of multivitamin preparations.

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