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Simultaneous analysis of vitamins B in pharmaceuticals and dietary supplements by capillary electrophoresis hyphenated with triple quadrupole mass spectrometry

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The advanced hyphenated method based on capillary electrophoresis (CE) on-line combined with electrospray ionization (ESI) and tandem mass spectrometry (MS/MS, here triple quadrupole, QqQ) was developed and applied in pharmaceutical analysis in this work. Under the optimized CE separation (background electrolyte: 50 mmol/L formic acid with pH 2.05), ESI ionization (sheath liquid: 0.1% v/v formic acid in 50% v/v aq methanol) and QqQ detection (100-160 V fragmentor voltage, 15-25 eV collision energy, depending on the type of analyte) conditions, highly efficient, reproducible, accurate, sensitive (sub μg/mL LODs) and selective (each analyte was unequivocally specified by two characteristic MS signals, i.e. qualifier, quantifier) determination of vitamins B was achieved. All vitamins B, namely thiamine, riboflavin, nicotinamide, nicotinic acid, pantothenic acid, pyridoxine, biotin, folic acid and cyanocobalamine, were simultaneously determined in various dosage forms (injection solution, tablets, and effervescent tablets) within ca. 16 min. The main benefits of the proposed CE-ESI-QqQ method in comparison with the CE-UV ones are considerably enhanced sensitivity and selectivity. Other benefits are low cost, simplicity, flexibility and environmental aspects when comparing CE-ESI-QqQ with HPLC-MS. Successful validation and application of the proposed CE-ESI-QqQ method suggest its routine use in highly effective and reliable multi-drug quality control.

1. Introduction

Vitamins are organic compounds essential for the human organism, which have to be supplemented from the diet because the organism is not able to synthesize them by its own. Vitamins B are soluble in the water and involve thiamine (B1), riboflavin (B2), nicotinic acid/nicotinamide (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9) and cyanocobalamine (B12), providing (individually or simultaneously) many important biological actions in organism. Vitamins B are included, mostly as mixtures, in many vitamin-fortified food products, food supplements, and pharmaceuticals serving in the auxiliary therapy for the different (mainly neurological) diseases. Hence, the simultaneous and highly reliable identification and determination of vitamins B is very important for the control of such products. On the other hand, it is problematic due to very different chemical structures of vitamins B, their instability and the complexity of the real matrices. From these reasons, a need of a powerful analytical method to control these vitamins simultaneously in pharmaceuticals as well as food supplements is obvious.

Internationally accepted conventional analytical methods based mainly on microbiological assay and immunoassay are usually laborious, time- and money-consuming and mostly do not allow the simultaneous analysis of several vitamins. On the other hand, recently highly efficient separation techniques including chromatographic (LC) and electrophoretic (CE) ones were applied for the simultaneous analysis of several vitamins in one run (Blake 2007). The LC-DAD/MS method was reported for the analysis of seven vitamins B in multivitamin dietary supplements (Chen and Wolf 2007; Chen et al. 2006). Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) with the UV detection were applied for the selected group of the vitamins B, namely B1, B2, B3, B5 and B6 in the pharmaceutical formulations (Fotsing et al. 1997 and 1999; Su et al. 2001; Gomis et al. 1999). Very recently, a simultaneous determination of eight vitamins B in food supplements by MEKC-UV method was reported (da Silva et al. 2013). The sensitivity of the UV detector, however, was lower for biotin and pantothenic acid due to lack of UV chromophores that is a main limitation in the use of this type of detection.

An on-line combination of CE separation with MS detection provides one of the most powerful tools in the analytical science. In some aspects it can be even more attractive as LC-MS due to high separation efficiency, flexibility, minimal sample and electrolytes consumption, water based working solutions, environmental aspect and relatively cost of CE instrument as well as its performance (Suntornsuk 2010; Bonvin et al. 2012; Stanova et al. 2010; Servais et al. 2006; Ahmed 2009; Ramautar et al. 2009; Pantuckova et al. 2009 and 2011). Here, the MS detection

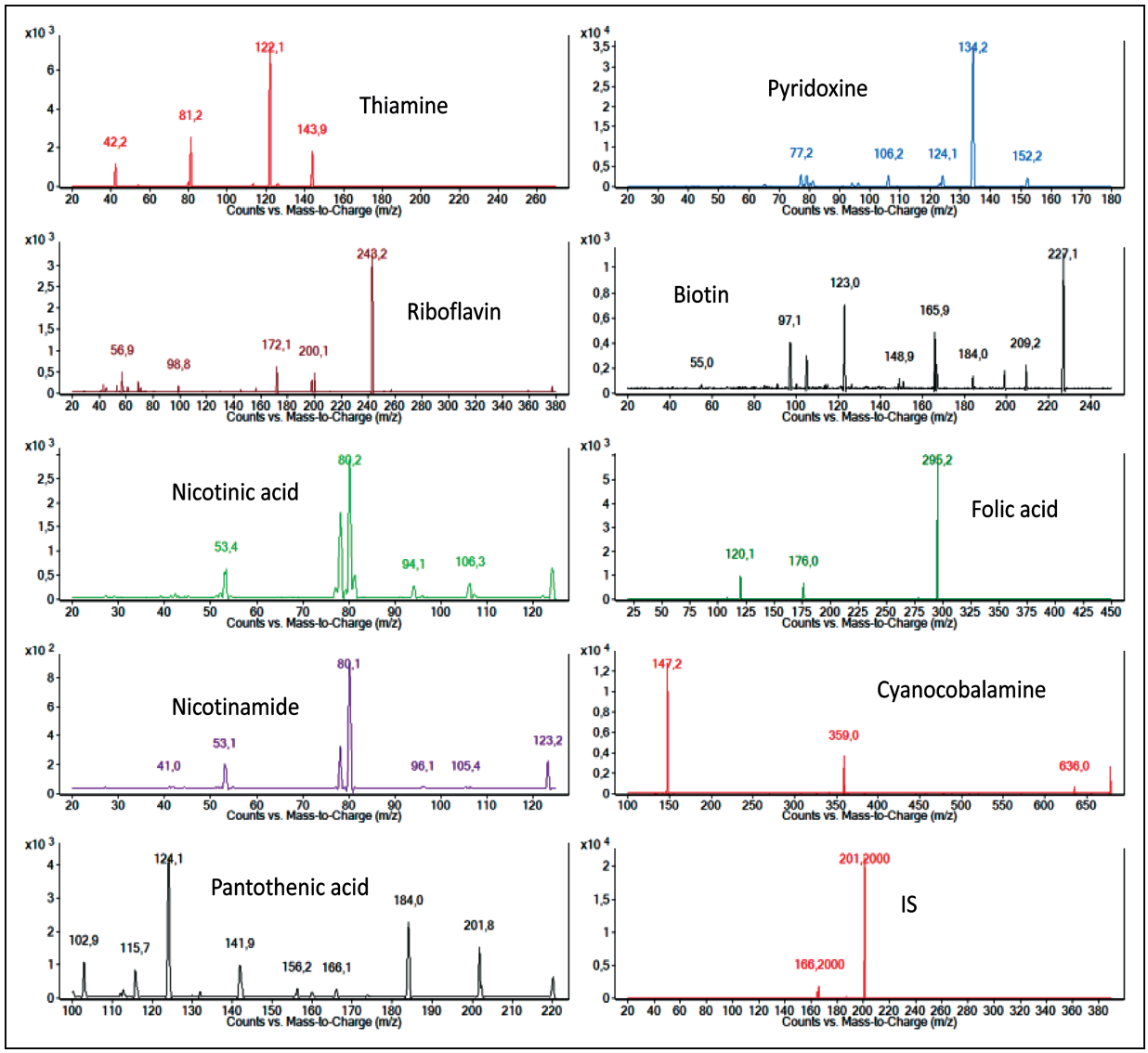

Fig. 1: Mass spectra of parent and product ions of vitamins B. The spectral profiles were used for the selection of product ions serving for the identification (qualifier) and quantification (quantifier) of the drugs (resulting data see in Table 1). The MS/MS spectra were obtained from the Product ion mode. The fragmentor voltage and collision energy were set for each drug as listed in Table 1. Concentrations of the drugs in the analyzed model samples were 20 μg/mL.

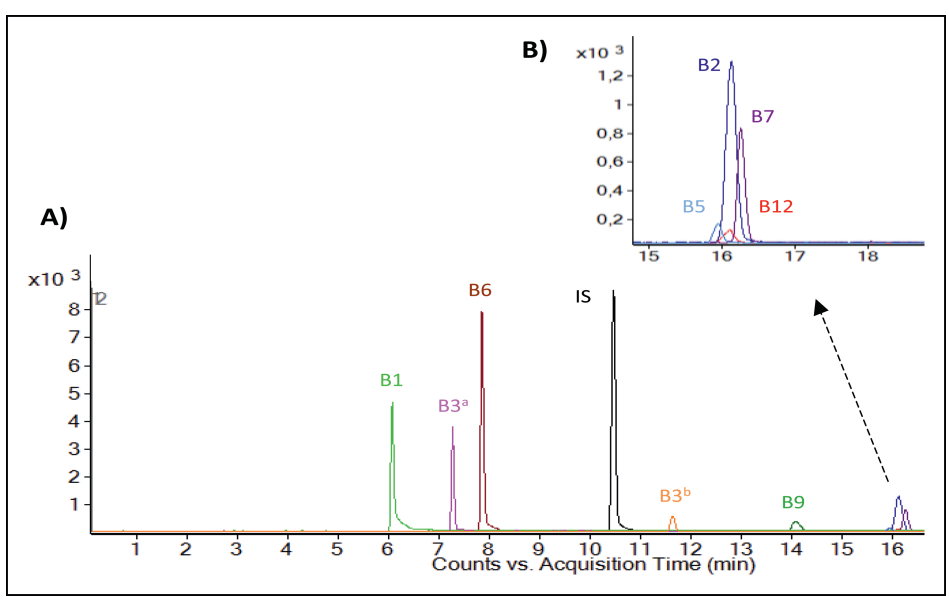

Fig. 2: Electrophoreogram from the simultaneous CZE-ESI-QqQ determination of vitamins B in the mixed standard solution (A) and the detail of the mixed peak migrating in the 16th minute (B). The concentration level of each vitamin as well as IS was 10 μg/mL. The parent-product ion transitions were set as given in Table 1. For the separation conditions see Experimental section. B1 – thiamine, B2 – riboflavin, B3[a] – nicotinamide, B3[b] – nicotinic acid, B5 – pantothenic acid, B6 – pyridoxine, B7 – biotin, B9 – folic acid, B12 – cyanocobalamine, IS – internal standard.

provides very high sensitivity (e.g. in comparison with the conventional detectors such as UV, regardless of the presence of a chromophore in the analyte) along with mass selectivity prerequisite for the unequivocal identification of the targeted analyte in a multicomponent mixture (that is impossible or problematic using UV). There have been only two papers (Tomas et al. 2010; Chen and Jiang 2008) published on the CE-MS analysis of individual vitamins B, but no full mixture analysis, so far.
The aim of the present work was to develop an advanced CE-MS/MS method for a simple, fast and highly reliable simultaneous identification and determination of eight vitamins B in various pharmaceuticals and dietary supplements, such as injections, tablets and effervescent tablets, useful for the routine use. Here, a tandem MS (MS/MS) such as triple quadrupole (QqQ) was employed in order to enhance the identification ability of the method and, by that, reliability of the results.

2. Investigations, results and discussion

2.1. Optimization of working conditions

The optimization process was aimed at all the three steps of the CZE-ESI-QqQ method, namely (i) CZE separation (composition, ionic strength and pH of the background electrolyte, BGE), (ii) electrospray ionization, ESI (composition and flow rate of the sheath liquid), and (iii) QqQ detection (fragmentor voltage, collision energy, nebulising gas pressure, drying gas flow rate and temperature, capillary voltage).
The volatile BGE buffers (formic, acetic acid) with low salt (ammonium formate, ammonium acetate) concentrations were needed for a proper compatibility of CZE with ESI. The effect of pH (studied in the wide range of 2.05-9.41) on the mobilities of analytes and EOF velocity was very pronounced. The best compromise between resolution, analysis time, reproducibility, dispersion, and detection sensitivity was obtained with BGE composed from 50 mmol/L aqueous formic acid with pH 2.05. Under these conditions the CZE separation was adequate for most of the vitamins and a rest co-elution (riboflavin, pantothenic acid, biotin and cyanocobalamine) was easily overcome by an additional resolution *via* the consecutive MS separation.
The CE-MS hyphenation was based on the ESI interface that is relatively robust and easy to implement. ESI ionization was performed in a positive ion mode. The sheath liquid composition (i.e. volatile matrix and additive providing its conductivity and improving the ionization of the analytes) and its flow rate were optimized to enhance the intensities of signals of analytes, compromising different ionization abilities of the analytes. A 50% v/v aqueous methanol solution containing 0.1% v/v formic acid was used as the optimum sheath liquid providing an acceptable compatibility with BGE. An 8 μL/min sheath liquid flow rate was optimal for achieving the stable separation current and maximum detection sensitivity.
Several operation modes of the MS software were chronologically applied to find the optimal parameters for the identification (*via* qualifier product ion) and quantification (*via* quantifier product ion) of the analytes (parent ions). The optimized fragmentor voltage and collision energy provided the optimum MS signals/characteristics of the parent and product ions, respectively, see data in Table 1 and MS spectra of the analytes in Fig. 1. Considering previous optimizations, the MRM (multiple reaction monitoring) mode was set on the optimum parent-product ion transitions and applied for the highly reliable and sensitive simultaneous determination of nine vitamins B in their model mixture, for the full CE-MS profile see Fig. 2 and for the extracted quantifier signals of the particular vitamins see Fig. 3. For the optimum performance of the MS detection, several working parameters related to the ESI-MS interface, such as nebulizing gas pressure, drying gas temperature and flow rate, capillary voltage, and protruding length of CE capillary from the injection sprayer, were optimized too (for the optimum settings see Section 3.1). Under these optimum conditions, nine vitamins B were analyzed in about 16 min with very good reproducibility of the migration times (less than 1 %) and maximum detection sensitivity.

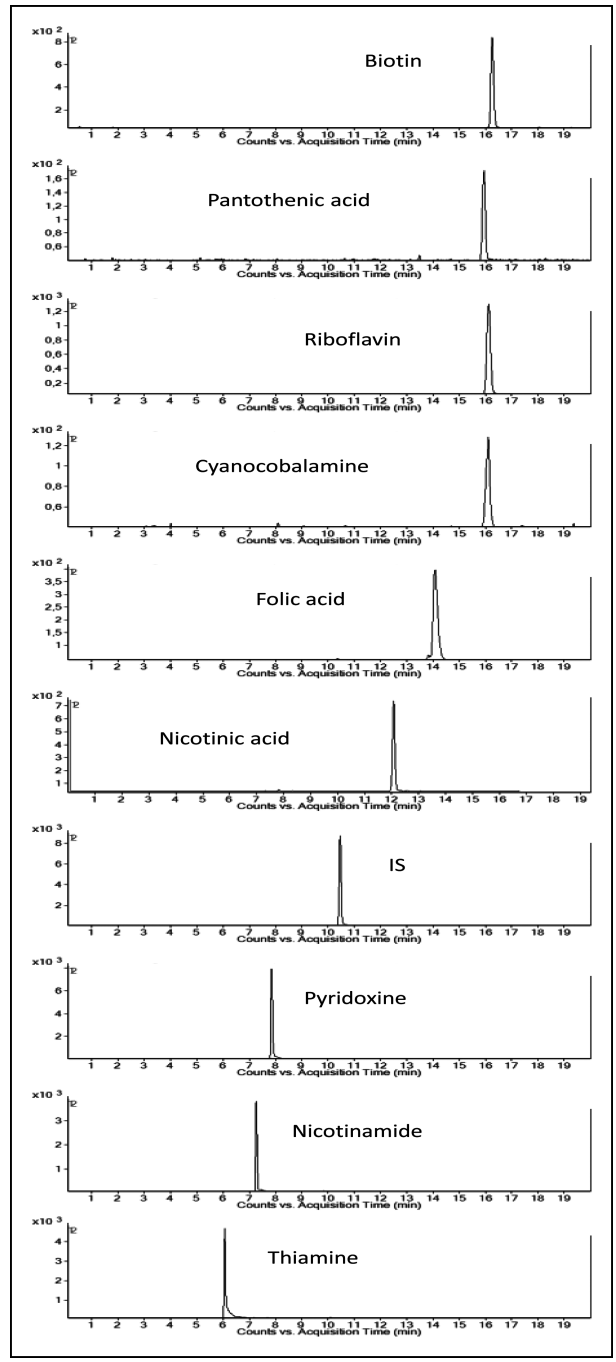

Fig. 3: MRM quantifier signals extracted from the simultaneous CZE-ESI-QqQ determination of nine vitamins B in the mixed standard solution (as in Fig. 2). The concentration level of each vitamin as well as IS was 10 μg/mL. The parent-product ion transitions were set as given in Table 1. For the separation conditions see Experimental section. IS – internal standard.

2.2. Validation of the method

The optimized CZE-ESI-QqQ method was validated and the resulting data are given in Table 2. The standard solutions of the vitamins (see Section 3.3.1.) were used for the validation

Table 1: Parent-product ion transitions set in the MRM mode

Analyte	Parent Ion	Fragmentor voltage [V]	Product Ion (quantifier)	Product Ion (qualifier)	Collision energy [eV]
Thiamine	265.2 $[M]^+$	100	122.1	81.2	25
Riboflavin	377.2 $[M+H]^+$	160	243.2	172.1	25
Nicotinamide	123.1 $[M+H]^+$	120	80.1	53.1	25
Nicotinic acid	124.1 $[M+H]^+$	160	80.2	53.1	25
Pantothenic acid	220.1 $[M+H]^+$	100	124.1	184.0	15
Pyridoxine	170.1 $[M+H]^+$	100	134.2	106.1	25
Biotin	245.2 $[M+H]^+$	100	227.1	123.0	20
Folic acid	442.2 $[M+H]^+$	100	295.2	176.1	25
Cyanocobalamine	678.4 $[M+H]^{2+}$	140	147.2	359	20
Cetirizine (IS)	389.3 $[M+H]^+$	120	201.1	166.1	20

Table 2: Performance parameters of the CE-ESI-QqQ method[a]

Analyte	$t_m \pm RSD_{tm}$ (min) ± (%)[b]	RSD_{area} (%)[b]	a (counts.min)	b (counts.min.μg $^{-1}$.mL)	r^2	LOD (μg/mL)	LOQ (μg/mL)	N	H (μm)
Thiamine	6.089 ± 0.32	3.95	0.0795	0.0293	0.9992	0.1047	0.3489	53179	15.43
Nicotinamide	7.301 ± 0.34	3.36	0.0383	0.0209	0.9994	0.0970	0.1617	76829	10.68
Pyridoxine	7.798 ± 0.35	1.44	0.0355	0.0506	0.9996	0.0419	0.1398	72267	11.37
Nicotinic acid	11.67 ± 0.26	3.66	−0.0435	0.0144	0.9958	0.1005	0.3432	77517	10.69
Folic acid	13.94 ± 0.55	4.44	−0.0098	0.0076	0.9993	0.1715	0.5716	30345	27.12
Cyanocobalamine	15.92 ± 0.61	3.44	−0.0056	0.0029	0.9988	0.2252	0.7506	48815	16.94
Riboflavin	15.96 ± 0.55	1.36	−0.0368	0.0294	0.9990	0.0852	0.2838	54947	14.99
Pantothenic acid	16.05 ± 0.57	3.85	−0.0043	0.0020	0.9983	0.7521	2.5070	96444	8.535
Biotin	16.09 ± 0.55	3.12	−0.0099	0.0117	0.9990	0.1001	0.3335	113498	7.26

[a] For the separation and other working conditions see the Experimental section. The vitamin standards were diluted to the concentration of 10 μg/mL and directly injected into the analyzer.
[b] RSD_{tm}, n = 6; RSD_{area} was calculated from the analyte/IS peak area ratios, n = 6.

experiments and the performance parameters given in Table 2 were evaluated according to the ICH guideline (ICH Harmonised Tripartite Guideline). Parameters of calibration lines were calculated by using Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

The limits of detection (LOD) and limits of quantitation (LOQ), calculated as the ratio of the signal-to-noise (S/N) to be 3S/N and 10S/N, respectively, were at sub μg/mL concentration levels that was favorable for the highly sensitive pharmaceutical analysis of the vitamins B. The determination coefficients (r^2) higher than 0.99 approved the acceptable linearities of the all calibration dependences tested in the concentration ranges given in Section 3.3.1. The precision, expressed relative standard deviation of (i) analyte/internal standard peak area ratio (RSD_{area}), and (ii) migration time (RSD_{tm}) of the analytes, reflected a very well optimized analytical procedure providing a high reproducibility of the measurements (RSD of migration times were less than 0.7%, RSD of peak area were less than 4.5 %). The recovery test, evaluated by comparing the spiked standard analyte signals in reference (water) and pharmaceutical (dosage forms) matrices at three different concentration levels (given in Section 3.3.1), indicated a negligible influence of the pharmaceutical matrices on the analytical signals of the studied vitamins (average recoveries were in the range of 96.33-102.7 %, except for biotin and folic acid tested in the eff. tbl. matrix) with a small influence of the eff. tbl. matrix on biotin and folic acid (average recoveries were slightly below 95%), see data in Table 3. The results from the recovery test approved acceptable accuracy of the proposed method. Favorable performance of the optimized CZE-ESI-QqQ method is obvious also from the good separation efficiency given by (i) the number of theoretical plates (N) as well as (ii) the height equivalent to one theoretical plate (H). The validation data and overall analysis time indicate the fast, efficient and highly reliable separation process useful for the routine use in the pharmaceutical analysis such as quality drug control. This CZE-ESI-QqQ method, in comparison with the previously published one (da Silva et al. 2013), provides improvements in analysis time, sensitivity and selectivity (each analyte was

Table 3: Determination of contents of vitamins B in pharmaceuticals by CE-ESI-QqQ method[a]

Pharmaceuticals	Declared[b] (mg)	Found[c] (mg)	Recovery[c] (%)
Milgamma inj.			
Thiamine	100	94.60 ± 0.63	98.38
Pyridoxine	100	99.50 ± 0.07	96.33
Cyanocobalamine	1	1.046 ± 0.94	101.9
B-Komplex forte tbl.			
Thiamine	15	14.58 ± 0.57	99.67
Riboflavin	15	12.44 ± 1.84	98.83
Nicotinamide	50	51.92 ± 2.75	102.7
Pantothenic acid	25	25.21 ± 1.68	100.7
Pyridoxine	10	10.77 ± 1.40	98.08
B-Komplex Generica eff. tbl.			
Thiamine	10	10.56 ± 1.75	101.6
Riboflavin	10	9.710 ± 0.51	99.85
Nicotinamide	50	52.68 ± 1.95	102.1
Pantothenic acid	35	35.59 ± 1.78	100.9
Pyridoxine	10	9.850 ± 1.50	99.52
Biotin	0.5	0.491 ± 1.90	93.19[d]
Folic acid	1	0.702 ± 0.50	93.08[d]

[a] For the separation and other working conditions see the Experimental section. For the preparations of the samples see section 3.3.2). The quantifier and qualifier ions of the vitamins (as given in Table 1) were set in the MRM mode and used for the calculations.
[b] Declared content corresponds with 2 mL of injection solution or with one tablet.
[c] ± RSD [%], n = 6; mean recovery was calculated from three concentration levels, n = 9.
[d] The particular spiked concentration levels and corresponding recoveries (in brackets) for biotin and folic acid were 2,5 μg/mL (92.86%), 5 μg/mL (93.05%), 7,5 μg/mL (93.66%), and 5 μg/mL (93.10%), 10 μg/mL (93.01%), 15 μg/mL (93.13%), respectively.

unequivocally proved by two MS signals, i.e. qualifier and quantifier) of the analysis.

2.3. Application of the method

The optimized and validated CZE-ESI-QqQ method was applied in the pharmaceutical analysis to simultaneously identify and determine the content of the all vitamins B in various commercial pharmaceuticals such as injections, tablets, and effervescent tablets, see the results in Table 3. The applications were carried out in the MRM mode. It is the preferred MS mode for the quantitation since it provides the best possible specificity (each vitamin was characterized by two independent signals, i.e. qualifier, quantifier) along with the highest signal-to noise ratio for given analytes (maximum intensity of detection response). Hence, very high reliability, selectivity (practically, signals without any interferences) and sensitivity of the determination was accomplished. The determined values obtained with the proposed method were consistent and in a good accordance with the declared ones. Only the levels of riboflavin in tablets and folic acid in the effervescent tablets were a bit lower than the declared ones. It can be due to several factors such as lower chemical stability, losses arising from storage and manufacture, etc., since the recoveries from the corresponding dosage forms showed acceptable accuracy of the proposed method.

It can be concluded that the proposed hyphenated CZE-ESI-QqQ method provides excellent analytical and application possibilities for the control of multi-drug pharmaceuticals such as vitamins B. It is needed especially in case of the dietary supplements where the differences between the declared and real contents of the active substances can fluctuate as a result of the absence of an effective analytical method for the post-production quality control of such products. The tandem MS detector, although more expensive, prevents any detection interferences in the real multicomponent matrices and offers superior sensitivity and specificity for the determination of vitamins B (including vitamins that do not contain UV sensitive chromophores, such as pantothenic acid and biotin) in comparison with UV detection. Moreover, the developed method (after its proper modification) has a great potential to be applied also for other multicomponent, e.g. biological matrices.

3. Experimental

3.1. Instrumentation

The capillary electrophoretic analyzer, namely Agilent 7100 Capillary Electrophoresis System (Agilent Technologies, California), was used in this work in a CE-ESI-MS/MS hyphenation for performing the CZE runs. Agilent ChemStation Softwave B.04.03 (Agilent) was used for the CE system operation and data acquisition. The CZE column was provided with a 50 μm I.D. uncoated fused silica capillary tube (Agilent) of a 820 mm total length (to ESI). As an optimum BGE 50 mmol/L formic acid (pH 2.05) was chosen. For better reproducibility, prior to the injection, the negative voltage -25 kV was applied for 30 s and the capillary was washed with BGE for 120 s. Washing of the capillary with 0.1 mol/L sodium hydroxide was necessary just at the end of the working day. The separating buffer was renewed in the vials after every three runs (for good reproducibility). The samples were injected hydrodynamically by applying a pressure of 50 mbar for 10 s. In order to ensure peak area reproducibility and a quantitative injection of the sample, after the injection of the sample, the short zone of BGE (50 mbar for 2 s) was introduced into the capillary. CZE analyses were carried out in the cationic regime of the separation (i.e. cathodic movement of the analytes) with the direct injections of the samples. A separation voltage of + 30 kV was progressively applied to the capillary. The resulting current was fixed at 10 μA. The experiments were performed in a constant temperature mode at 20 °C.

A mass spectrometer Agilent 6410 Series Triple Quadrupole (Agilent) was used with a triple quadrupole MS tandem (QqQ) equipped with an electrospray ionization source (ESI). Mass Hunter Work Station B.03.01 (Agilent) was used for the MS system operation and data acquisition. The CE-MS coupling was carried out using a sheath liquid coaxial interface (Agilent). The sheath liquid was delivered by a pump Agilent 1260 Infinity (Agilent) and flowed through a splitter set at a ratio of 1:100 into the sprayer. The sheath liquid (0.1% v/v formic acid in 50% v/v aq methanol) was co-injected with a syringe pump at a flow rate of 8 μL/min. The nebulizing gas (N_2) flows through the outer capillary channels under the pressure set to 10 psi. The drying gas temperature was 300 °C and its flow rate was 5 L/min. The capillary voltage of + 5000 V was set in MS detector. The optimal protruding length of the CE capillary from the interface was about 0.2-0.3 mm.

3.2. Chemicals and samples

The electrolyte and sheath liquid solutions were prepared from the chemicals (formic and acetic acid, ammonium formate, ammonium acetate, ammonia solution and methanol) obtained from Sigma-Aldrich (Steinheim, Germany) in ultrapure water for CE purchased from Agilent. The used chemicals were of analytical or LC-MS (formic acid, ammonium acetate and formate, methanol) grade. The solutions of electrolytes were filtered before the use through disposable membrane filters of a 0.45 μm pore size (Millipore, Molsheim, France).

The vitamin standards, namely thiamine hydrochloride (B1), riboflavin (B2), nicotinic acid and nicotinamide (B3), calcium D-pantothenate (B5), pyridoxine hydrochloride (B6), biotin (B7), folic acid (B9), cyanocobalamine (B12), and cetirizine dihydrochloride (serving as internal standard) were purchased from Sigma-Aldrich.

The commercial pharmaceuticals tested (Milgamma injection solution, B-Komplex forte tablets and B-Komplex Generica effervescent tablets) were obtained from a local drug store.

3.3. Procedures for sample and standard solution preparations

3.3.1. Standard solutions

The standard stock solutions of vitamins B and the internal standard (IS) were prepared by dissolving 10 mg of the reference substances in 10 mL of ultrapure water, with exception of B2, B7 and B9 prepared in 10 mL amber volumetric flasks by dissolving 10, 20 and 20 mg of powder, respectively, in 500 μL of ammonia solution (26% g/v) and aq. formic acid solution with the final pH 7.00. All stock solutions were protected from the light in the dark glass flasks and stored in the refrigerator at 4 °C. The working solutions were prepared by the appropriate dilutions of the stock solutions with ultrapure water or by spiking the dosage forms with the stock solutions. The concentration of IS in each working solution was 10 μg/mL.

The working standard solutions prepared in the concentration range of 6.250-100 μg/mL (thiamine, nicotinamide and pantothenic acid) and 3.125-50 μg/ml (all other vitamins), served as the model calibration solutions. Each calibration point (analyte/IS peak area ratio *vs.* analyte concentration) was measured three times.

For the recovery experiments, the real matrices (dosage forms) were spiked by the standards of vitamins (according to their appearance in the particular commercial dosage forms) in three concentration levels (50, 100, and 150% of the estimated value of vitamin) and the resulting peak areas were compared to those obtained at the same levels in the water (reference) matrix.

3.3.2. Samples preparation

Milgamma injection solution was just appropriately diluted to ca. 10 μg/mL concentrations of the vitamins and directly injected to the analyser.

Six tablets of B-Komplex forte tbl. and B-Komplex Generica eff. tbl., respectively, were ground to a homogenous powder and the quantity of the powder corresponding to one average tablet weight was transferred into a 50 mL amber volumetric flask and dissolving in 500 μL of ammonia solution (26% g/v) and aq. formic acid solution with the final pH 7.00. The mixtures were ultrasonicated for 5 min. The resulting solutions were appropriately diluted with the water prior to the analysis and filtered by a 0.45 μm pore size syringe filter (Millipore) before the injection into the CE equipment.

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