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Article

Simultaneous Analysis of Biologically Active Pyridines in Pharmaceutical Formulations by Capillary Zone Electrophoresis

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A capillary zone electrophoretic method using UV detection is developed for the analysis of four biological active pyridines [i.e., nicotine (NIC), cotinine (COT), nicotinic acid (NA), and nicotinamide (NM)]. The separation of the pyridines is achieved in 25 mM sodium dihydrogen phosphate (pH 2.1) using a fused-silica capillary with an effective length of 56 cm and an inner diameter of 50 μm (extended light path), hydrodynamic injection at 50 mbar for 10 s, a temperature of 25°C, applied voltage of 30 kV, and UV detection at 260 nm. These conditions provide baseline separation of all the analytes [resolution (R_s) > 3.6] in 9.4 min with good linearity (r^2 > 0.998, in ranges of 50–600 μg/mL for NIC, 8–160 μg/mL for NM, and 10–200 μg/mL for COT and NA), precision (relative standard deviation <2.04%), recovery (96.4–101.6%), limits of detection (<3.0 μg/mL), and quantitation (<10 μg/mL). The method is robust upon the alterations of pH of BGE, separating voltage, and injection time [the RSDs of the relative migration time (migration time of the analyte/migration time of the internal standard) and resolution <3.26%]. The method is efficient, reliable, and simple for the routine analysis of NIC, NA, and NM in various products such as gum and tablets and can be applied to determine COT in thermal degradation of NIC gum.

Introduction

Pyridines are heterocyclic aromatic organic compounds, which are present in many biologically active substances, including nicotine (NIC), cotinine (COT), nicotinic acid (NA), and nicotinamide (NM) (Figure 1). NIC is a major alkaloid present in tobacco, which accounts for 98% of total alkaloids and is the major known addictive component in tobacco smoke (1–3). NIC is metabolized to more than 20 different derivatives; among them, COT is a primary metabolite (70–80%) with a similar structure to NIC (1). NIC and COT are frequently used as biomarkers of tobacco exposure (4, 5). Additionally, NIC has been added to pharmaceutical formulations including chewing gums, sprays, inhalers, and beverages as smoke cessation products. Thus, the detection of NIC content is important for both pharmaceutical and food industries. The United States (USP) and British Pharmacopeias (BP) recommend non-aqueous titration for the assay of NIC in raw material (6, 7), whereas high-performance liquid chromatography (HPLC) is for the assay of NIC in gum (6). Additionally, the analyses of NIC and COT in different matrices by several methods have been described in literature, such as HPLC (8–15), gas chromatography (GC) (16–23), and capillary electrophoresis (24–31). Most of these works focused on the analyses of NIC, its related alkaloids, and metabolites in biological samples (e.g., urine, plasma, saliva, brain tissue, human milk, hair, vegetables, and food products), tobacco, or cigarette smoke (1, 2, 8–28). Thus, sample pretreatment [e.g., liquid–liquid extraction, solid-phase extraction (SPE), solid-phase microextraction (SPME), microdialysis or cloud point extraction] and sensitive detectors such as mass spectrometer (MS), tandem MS, and light emitted diode induced fluorescence are required for the detection of NIC and its derivatives in trace amounts. One paper described the analysis of NIC in bulk material, as well as extended and immediate release dosage forms (3), and another paper describes the analysis of five NIC related alkaloids in chewing gums, beverage, and tobacco (30).

NA (niacin) and NM (niacinamide) belong to the vitamin B group, which are water-soluble vitamins (32, 33). They are well–known precursors in the synthesis of the several co-enzymes, which are involved in cell metabolism, such as nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Deficiency of these vitamins causes inflammation of mucus membranes and an illness known as pellagra (34). In addition, NA is clinically used for the treatment of dyslipidemia, which favorably affects all of the lipoprotein risk factors in atherosclerotic diseases (33). Therefore, determination of NA and NM in supplements, nutraceuticals, and pharmaceuticals becomes necessary from pharmacological and pharmaceutical aspects. The USP suggests spectrophotometry for the assay of NA in raw material and injection, and HPLC for assay of NA in tablets, NM in raw material, injection, and tablets (6). BP recommends aqueous titration for the assay of NA in raw material and tablets, and non-aqueous titration for the assay of NM in raw material (7). Previous works on the analyses of NA and NM by HPLC have been reported (35–40). UV and diode array detectors (DAD) were mainly used for the determination of NA and NM in multivitamin preparations, whereas SPE coupled with MS was employed for the detection of both pyridines in industrial effluent (38) and in plasma (35, 36, 39, 40).

Because of the related structures of NIC, COT, NA, and NM (Figure 1), it is very interesting to establish a fast, simple, and cost effective method for the analysis of these compounds. Among several methods, CE has a high potential due to its advantages in terms of applicability for a wide range of compounds, high separation efficiency, simplicity, low solvent consumption, and short analysis time. Up to the present, there is no CE method for the simultaneous analysis of these pyridines. The current work is aimed to develop a common CZE method for the separation of NIC, COT, NA, and NM and to apply the method for the analysis of these analytes in pharmaceutical formulations.

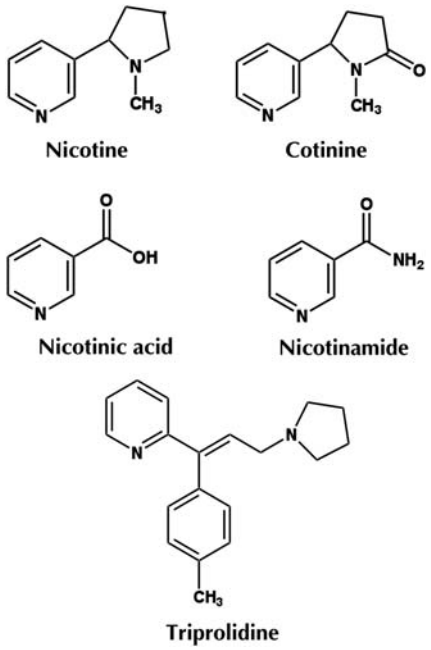

Figure 1. Structures of nicotine, cotinine, nicotinic acid, nicotinamide and triprolidine (IS).

Experimental

Chemicals and reagents

NIC and anhydrous citric acid were purchased from Riedel-de Haën (Seelze, Germany). COT was from Fluka (Buchs, Switzerland). NA, NM, triprolidine (TRI), and sodium dihydrogen phosphate were from Sigma-Aldrich (St. Louis, MO). NIC chewing gum (2 and 4 mg/gum), NA tablet (50 mg/tablet), a multivitamin tablet containing NM (15 and 20 mg/tablet), and a multivitamin tablet containing NM and NA (45 and 15 mg/tablet) were obtained from local drugstores. MeOH was from Labscan Asia (Bangkok, Thailand). All reagents were of analytical grade. Water was water for irrigation from Thai Otsuka (Samutsakorn, Thailand).

Instrumentation

CE separation was performed on a ^{3D}CE instrument model G1600A (Agilent Technologies, Waldbronn, Germany) and controlled by a PC through Agilent ChemStation Plus software version A.08 (G1601A). Separations were carried out using fused-silica capillaries with a total length (L) of 64.5 cm, an effective length of 56 cm, and an inner diameter (i.d.) of 50 μm (extended light path) from Polymicro Technologies (Phoenix, Arizona). All experiments were performed in positive polarity voltage (anodic site at the inlet and cathodic site at the outlet). Detection was by a diode-array detector using a detection wavelength at 260 nm. For daily use, the capillary was rinsed with 1 M sodium hydroxide, 0.1 M sodium hydroxide, water, and BGE for 5 min, respectively. Between runs, the capillary was rinsed with 0.1 M sodium hydroxide, water, and BGE for 2, 2, and 3 min, respectively. The BGE could be kept in a refrigerator for 7 days.

Standard and sample preparations

Stock solutions of NIC, COT, NA, NM, and TRI [as an internal standard (IS)] were separately prepared by dissolving 10 mg of each standard in 10 mL water (1.000 μg/mL). The stock solutions were protected from light, stored at 2°–8°C and used within 7 days. Working standard solutions were diluted from the stock solutions with water to obtain 1–1.000 (NIC), 3–200 (COT), 2–200 (NA), 2.5–160 (NM), and 100 (TRI) μg/mL.

Sample solutions were freshly prepared prior to CE analyses. Extraction procedures for nicotine gum were modified (30). Nicotine gum was cut into small pieces and transferred to a 15-mL centrifuge tube. Ten milliliters of methanol, containing 100 μg/mL of TRI, was added into the tube and mixed in a shaker at 65°C for 40 min. After cooling down to room temperature, the mixture was sonicated for 15 min and centrifuged at 6,000 rpm for 10 min. A clear solution was evaporated and reconstituted with 10 mL of water. Twenty NA tablets were triturated and powder equivalent to 500 mg of NA was dissolved with water, heated (30 min), sonicated (2 min), shaken (15 min), and adjusted to 100 mL. Ten NM tablets were triturated and powder equivalent to 25 mg of NM was dissolved with water, heated (30 min), and adjusted to 250 mL. TRI solution (as an internal standard) was added into the NM and NA sample solutions, and the solutions were diluted to 50 μg/mL (for NM) and 10 μg/mL (for NA). All solutions were filtered through a 0.2 μm membrane filter and degassed for 10 min prior to CE analyses.

Forced degradation of NIC gum by thermal degradation was modified (41). NIC gum was cut into small pieces, transferred to a test tube, and left in a water bath with the controlled temperature at 90°C under atmospheric pressure for 6 h. The sample was extracted using the procedure described earlier, except the reconstituted volume was 2.5 mL.

Method development and validation

The CE separation of NIC, COT, NA, and NM was optimized by varying between citric acid and sodium dihydrogen phosphate and by altering the pH and concentrations of BGE. In addition, the applied voltage and the injection times were varied. A detection wavelength at 260 nm was selected because all the analytes showed maximum UV absorption around this wavelength. The optimized CE conditions were determined from resolution (R_s), tailing factor (TF), number of theoretical plates (N), and relative standard deviations (RSD) of the relative migration time (t_r, calculated from the ratios of the migration time of the analyte, and the migration time of the internal standard) and the peak area ratios. Identification of migration order of the resolved compounds (NIC, COT, NA, and NM) was confirmed by running the electropherograms of the individual pure compound under identical separation conditions.

The optimized CZE conditions were validated in terms of linearity, precision, recovery, limits of detection (LOD), and quantitation (LOQ). Calibration curves of NIC (50–600 μg/mL), NM (8–160 μg/mL), and COT and NA (10–200 μg/mL) were established by plotting the peak area ratios against five different concentrations of the individual analyte. TRI (as the internal standard) was added into the calibrating solutions to obtain the concentration of 100 μg/mL. Triplicate injections were made

for each concentration. The correlation coefficients (r^2), response factors, and the residual of the regression line (calculated from the residual standard deviation) were calculated by Microsoft Office Excel (version 2003). Method precision, calculated from the RSDs of t_r and the peak area ratios, was determined from an injection repeatability ($n = 10$) [within-day ($n = 3$), and between-day ($n = 6$)] assay of the three different concentrations of the working standard solutions. Recovery of the method was estimated by spiking different concentrations (80, 100, and 120% percent of nominal concentrations) of NIC, NM, and NA into the sample solutions ($n = 3$). The nominal concentrations were 200, 10, and 50 μg/mL for NIC, NM, and NA, respectively. The method robustness was evaluated by changing of pH (± 0.2 pH unit), voltage (−3 and −5 kV), and the injection time (± 1 s) from the optimum condition. The RSDs of t_r and R_s were calculated. The LOD and LOQ were based on signal-to-noise (S/N) ratio of 3 and 10, respectively.

Results and Discussion

Method development

TRI was an appropriate internal standard because it was well-separated from NIC, COT, NM, and NA under all investigated conditions. The initial separation of the analytes was performed in 150 mM citric acid and 25 mM sodium dihydrogen phosphate (pH 2.1, 2.3, and 2.5) (27, 31). All the analytes and TRI were well-separated ($R_s > 2.0$) in both BGE with a migration order of NIC, NM, COT, and NA, respectively. However, the peaks of NM and NA showed tailing (TF > 1.4) in the citric acid solution. Sodium dihydrogen phosphate buffer (pH 2.1) was selected for further optimization, because it provided better peak shape, and the S/N from this buffer was 1.3-fold higher than in the citric acid solution. Varying the pH from 1.9 to 3.5 greatly influenced the migration time and the peak shape of NA. From pH 1.9 to 2.9, migration times of NIC, NM, and COT remained almost constant within 7 min, but that of NA increased from 11.2 to 24.9 min. These results are in good agreement with those reported by Marsh et al. (28) and Terekhova et al. (29), which confirms that low pH (between 2.0 and 2.7) should be maintained to keep NIC, COT, and NM in their protonated forms. However, NA showed distinct behavior due to the carboxylic group in its molecule, which was deprotonated at pH from 2.5 to 3.5, resulting in an asymmetric peak (TF = 0.7). Moreover, the separation efficiency of all the analytes significantly dropped at a pH greater than 2.5. For example, N dropped from 26.5 to 7.9×10^4 for NIC, from 8.9 to 2.8×10^4 for COT, from 13.4 to 3.8×10^4 for NM, and from 6.9 to 2.8×10^4 for NA when pH increased from 2.5 to 2.7. At pH 3.5, COT and NA co-migrated ($R_s = 0$). Although the highest resolution was obtained at pH 2.5 ($R_s > 5.9$), pH 2.1 was chosen as it offered the lowest RSD of t_r (<0.37%) and highest N (>6.9×10^4) within 10.8 min (comparing to 17.1 min at pH 2.5). Results from effects of BGE concentrations revealed that increasing the sodium dihydrogen phosphate buffer (pH 2.1) concentrations from 15 to 30 mM increased migration time from 8.0 to 13.9 min. Increased resolution was also observed due to reduction of electro-osmotic flow (EOF). However, increasing the BGE concentration to 30 mM increased the RSDs of t_r up to 4.5% because of the high current causing elevated temperature and peak dispersion. Phosphate buffer at 25 mM was optimal as it gave the lowest RSD of t_r for most analytes (<0.37%).

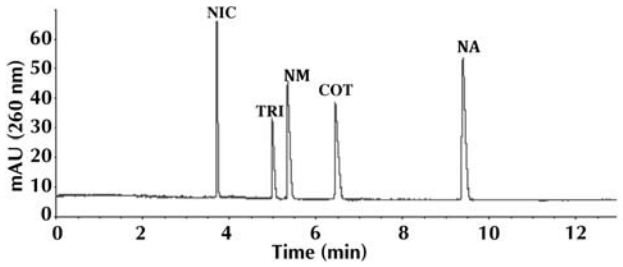

Figure 2. Electropherograms of the analytes and the internal standard (100 g/mL) under the optimum conditions. Conditions: 25 mM sodium dihydrogen phosphate (pH 2.1); capillary, 64.5 cm total length (8.5 cm to the detector), 50 μm id (extended light path capillaries); hydrodynamic injection at 50 mbar for 10 s; temperature 25°C; voltage, 30 kV; detection by UV absorbance at 260 nm. Peak identification: NIC = nicotine, TRI = tripolidine, COT = cotinine, NA = nicotinic acid and NM = nicotinamide.

Table I
Analytical Parameters of the Analytes Under the Optimized CE Condition*

	t_r	μ_e (× 10^{-8}/m^2/V/s)†	$w_{0.5}$ (min)‡	N (× 10^4)§	R_s††
NIC	0.75	4.48	0.03	11.4	20.1 (NIC/TRI)
NM	1.07	2.82	0.06	3.78	3.6 (TRI/NM)
COT	1.29	2.17	0.08	3.45	8.9 (NM/COT)
NA	1.87	1.20	0.08	6.76	21.0 (COT/NA)

*t_r = relative migration time calculated from t_m/t_{IS}.
†μ_e = electrophoretic mobility.
‡$w_{0.5}$ = peak width at 50% of peak height.
§N = number of theoretical plates.
††R_s = resolution.

Table II
Analytical Figures of Merit*

	Response factor (μg/mL)	Precision (%RSD)						LOD† (μg/mL)	LOQ‡ (μg/mL, $n = 3$)
		Injection ($n = 10$)		Within-day ($n = 3$)		Between-day ($n = 6$)			
		t_r	Area ratio	t_r	Area ratio	t_r	Area ratio		
NIC	100.0 (0.00)	1.82	0.58	0.84	0.62	2.04	1.64	1.0	3.2 (0.88)
NM	73.5 (2.35)	1.55	1.86	0.93	1.83	1.80	1.62	2.0	5.5 (1.80)
COT	82.0 (0.73)	1.78	1.32	1.49	1.26	1.61	1.32	2.5	8.0 (1.28)
NA	25.1 (1.29)	1.66	1.66	1.13	0.77	1.12	0.98	3.0	10.0 (1.80)

* t_r = relative migration time calculated from t_m/t_{IS}.
† LOD = limit of detection.
‡ LOQ = limit of quantitation, numbers in parentheses represent %RSDs.

Table III
Recovery data

	% Recovery				
%Add	80	100	120	Average	%RSD ($n = 3$)
NIC	95.4	96.6	97.2	96.4	0.98
NM	98.2	100.5	101.5	100.1	1.66
NA	100.4	102.4	102.0	101.6	1.06

Table IV
Robustness Data*

	pH				Voltage (kV)				Injection time (s)			
	1.9	2.1	2.3	%RSD	25	27	30	%RSD	9	10	11	%RSD
t_c												
NIC	0.78	0.75	0.75	2.28	0.74	0.74	0.75	0.78	0.76	0.75	0.79	2.72
NM	1.09	1.07	1.08	0.93	1.07	1.05	1.07	1.09	1.06	1.07	1.07	0.54
COT	1.26	1.29	1.28	1.20	1.30	1.27	1.29	1.19	1.27	1.29	1.26	1.20
NA	1.93	1.87	1.93	1.81	1.86	1.84	1.87	0.82	1.91	1.87	1.91	1.22
R_s†												
NIC/TRI	19.9	20.1	20.8	2.33	20.9	20.5	20.1	1.95	21.1	20.1	19.9	3.16
TRI/NM	3.5	3.6	3.6	1.62	3.50	3.6	3.6	1.62	3.6	3.6	3.5	1.62
NM/COT	8.6	8.9	8.7	1.75	8.7	8.5	8.9	2.30	9.0	8.9	8.6	2.36
COT/NA	21.9	21.0	22.4	3.26	20.4	20.0	21.0	2.46	21.3	21.0	20.1	3.00

* t_r = relative migration time calculated from t_m/t_{IS}.
† R_s = resolution.

Increasing the voltage from 20 kV to 30 kV decreased the migration time from 22.6 to 9.4 min due to the enhanced EOF velocity. At a lower voltage (20 kV), most peaks were broad with lower S/N. A voltage of 30 kV was selected because it gave the shortest t_m (9.4 min). Sample loading effects (50 mbar for 5–20 s) showed that 10 s was appropriate because the loading time of 5 s provided peaks with lower sensitivity, whereas at injection times of 15 and 20 s, the NA peak was highly skewed and broad (TF = 1.7) with significant drops of separation efficiency of most analytes. The optimum separation of the analytes was in 25 mM sodium dihydrogen phosphate buffer (pH 2.1) using voltage of 30 kV and injection time of 50 mbar for 10 s (Figure 2 and Table I).

Table V
Assay Data*

	Brand	t_r (min, $n = 9$)	% Labeled amount ($n = 3$)	USP 31 limit
NIC (2 mg/gum)	A	0.75 (1.23)	103.3 (1.03)	90.0–120.0
NIC (2 mg/gum)	B	0.75 (1.69)	102.9 (0.79)	
NIC (4 mg/ gum)	B	0.76 (1.87)	100.9 (1.01)	
NM (15 mg/tablet)	C	1.09 (1.56)	101.4 (1.30)	90.0–110.0
NM (20 mg/tablet)	D	1.08 (1.21)	100.2 (1.52)	
NA (50 mg/tablet)	E	1.84 (1.43)	99.1 (1.03)	90.0–110.0
NM, NA (45 and 15 mg/tablet, respectively)	F	1.08 (1.85), 1.93 (1.42)	104.3 (1.86), 103.7 (1.95)	

*t_r = relative migration time calculated from t_m/t_{IS}, numbers in parentheses represent %RSDs.

Method validation

The linearity, precision, recovery, robustness, LOD, and LOQ of the proposed method were evaluated. Detector response was linear with r^2 between 0.998–0.999 in ranges of 50–600 μg/mL for NIC, 8–160 μg/mL for NM and 10–200 μg/mL for COT and NA. Response factors (1/slope) are in the range of 100.0–25.1 μg/mL, and their RSDs varied from 0.00–2.35% (Table II). The residuals of the regression lines ranged from 1.61 to 4.67. Injection, within-day, and between-day precision showed RSDs of <2.04% and <1.86% for t_r and the peak area ratios, respectively (Table II). The LODs were less than 3.0 μg/mL, and the LOQs were less than 10 μg/mL (RSDs of <1.80%) (Table II). The mean recoveries of NIC, NM, and NA were 96.4, 100.1, and 101.6, respectively, with RSDs of <1.66% (Table III). The recovery data indicates that the sample matrices did not affect the quantitation of the investigated analyte in the samples. The robustness data revealed that t_r and R_s were not significantly affected despite the varied pH of the BGE, separating voltage, and injection time (Table IV). The RSDs of these analytical parameters were between 0.54% and 3.26%. Thus, the obtained results indicate that the method is reasonably robust. In general, the validation data shows that the method was appropriate for the analysis of the investigated biologically active pyridines in pharmaceutical formulations.

Applications

Applications of the proposed method were demonstrated in the assay of pharmaceutical products containing single and combined biologically active pyridines in formulations and in stress tests of a product. The method was applied to determine NIC in three different lots of NIC gum, NM in two different lots of vitamin B complex tablets, NA in one lot of NA tablets, and NM and NA in one lot of multivitamin tablets. BGE and water (sample solvent) did not interfere with the CE separation because no extra peaks were observed during the analysis. Relative migration times of the analytes in the standard (Table I) and sample solutions (Table V) are comparable. The internal standard plays a role on precision enhancement. In the sample solutions, the data shows that RSDs ($n = 9$) calculated from relative migration times (1.93%) were smaller than those calculated from migration times (3.21%). Percent label amounts of the tested samples were within 99.1–104.3% (RSD < 1.86%), which were within the USP limits (Table V). Figure 3 shows typical electropherograms of NIC, NM, and NA in various products. Additionally, the proposed method could be applied for determination of COT in forced degradation of NIC gum. The degradant (COT) was well separated from the major peak (NIC). Small amounts of COT (1.48% w/w) were found in the thermal degradation (90°C, 6 h) NIC gum sample, and no other peaks were observed within 10 min (Figure 3B).

This is the first time that simultaneous separation of these biologically active pyridines by CZE was demonstrated. Most previous works demonstrated the separation of NIC and its metabolites, NIC alkaloids, tobacco alkaloids (25–32). Additionally, no work on CE of NM and NA is demonstrated, only HPLC methods are reported (36–41). Validation data of the method provides better dynamic range and precision than reported (30, 31). Unlike other work, the current method

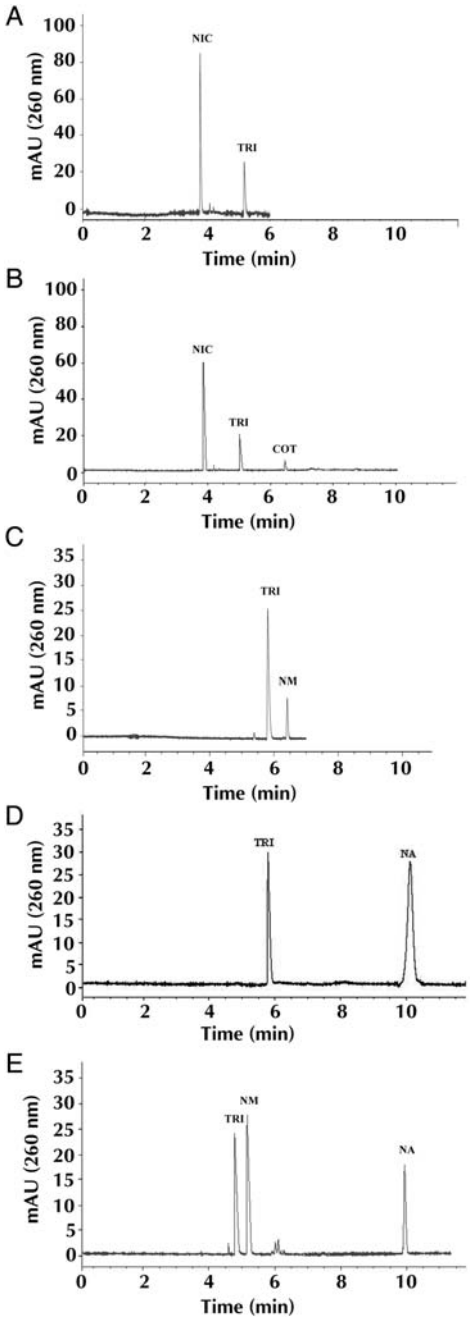

Figure 3. Electropherograms of (A) nicotine in chewing gum (B) nicotine and its oxidized product (cotinine) from stress test (C) nicotinamide in multivitamin tablets (D) nicotinic acid in tablets and (E) nicotinamide and nicotinic acid in multivitamin tablets. Conditions and peak identification: same as Figure 2.

shows applications in real samples both in regular drug formulations and in stress test samples.

Conclusion

The CZE method for the simultaneous analysis of NIC, COT (in stress test of NIC gum), NM, and NA in pharmaceutical formulations was established. Comparing to previous reports (3, 30, 31), the proposed method provided better precision and a wider dynamic range. Moreover, the method is efficient, simple, and rapid, which could be applied to assays of NIC, NM, and NA in formulations. Sample preparation is minimal, which required a simple liquid extraction for NIC gum and diluting with water for NM and NA tablets. In the cases of the NM tablets and the combined NM and NA formulations, the presences of vitamin B complexes (e.g., B1, B6, B2, and B12), vitamin C, calcium pantothenate, and biotin did not show any interfering peaks during the observed analysis time (15 min). The recovery data also confirms that the method is selective and not affected by the sample matrices. The short analysis time allowed a high throughput analysis of up to 16 samples/h (based on the migration time of NIC). From economic and practical aspects in the pharmaceutical industry, it is desirable to have a common method that can be used for quality control and a stability study of various active pharmaceutical ingredients, either in single or combined formulations.

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