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Fast Separation and Determination of Four Tobacco-Specific Nitrosamines in Cigarette Smoke Using UPLC-MS/MS

XIAO-LAN LI[1], YUN ZHOU[1], ZHI-YAN CHEN[1], QI-BIN LIU[1], WEN-JING ZHU[2], GUI-FENG HUANG[2] and JUN YANG[2,*]

[1]Technology Center, China Tobacco Guangxi Industrial Co. LTD., Nanning 530001, P.R. China
[2]Research Center of Tobacco and Health, University of Science and Technology of China, Hefei 230052, P.R. China

*Corresponding author: Tel/Fax: +86 551 63492065; E-mail: yjun8202@ustc.edu.cn

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An ultra high pressure liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method for fast separation and determination of the four major tobacco-specific nitrosamines in mainstream cigarette smoke has been developed. Cigarette smoke particulate matter was extracted with 100 mM ammonium acetate after addition of isotopically labeled internal standards, further purified by solid-phase extraction using PCX column. Subsequently, the target analysis was performed by UPLC-MS/MS. The limit of quantification (S/N > 10) for the four tobacco-specific nitrosamines were 0.23, 0.29, 0.25 and 0.35 ng/mL respectively, with a linear calibration range spanning 0.8-100 ng/mL. This method was validated by comparing the quantitative results for tobacco-specific nitrosamines with the conventional standard method.

Keywords: Tobacco-specific N-nitrosamines, UPLC-MS/MS, Cigarette smoke, Solid-phase extraction.

INTRODUCTION

Tobacco-specific nitrosamines (TSNAs), such as N-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB), are the most important and potential carcinogens identified in both tobacco and cigarette smoke[1,2]. Because of health concerns, there has been a long standing interest to develop a sensitive, fast and robust analytical method to determine tobacco-specific nitrosamines in cigarette smoke. Several analytical methods, involving gas chromatography (GC)[3], high-performance chromatography (HPLC)[4], gas chromatography-mass spectrometry (GC-MS)[5], and gas chromatography coupled with thermal energy analyzer (GC-TEA)[6], have been reported so far. Among all of the methods, GC-TEA which provides reasonable sensitivity and is relatively specific for nitrosamine compounds, is the most widely used method for determination of tobacco-specific nitrosamines in cigarette smoke. Recently, developed HPLC-MS/MS methods have been used for determining tobacco-specific nitrosamines[7,8]. In comparison with GC-TEA method, it provides higher selectivity, significantly lower limits of detection and a greater linear dynamic range. However, an important problem to be faced with in method development, validation, and routine use of HPLC-MS/MS is matrix effects[9-11], especially using an electrospray ionization (ESI).

There have been several operational strategies employed to minimize or compensate the interferences of matrix components. One way is to apply cleanup procedures[12] prior to HPLC-MS/MS analysis, which can help decrease the introduction of co-extracted matrix compounds into the analytical system. Another way to effectively compensate for signal suppression or enhancement is to use stable isotope-labeled internal standards[7,13] which are chemically and structurally the same as the target analytes and just different in molecular mass. Besides, optimization of chromatographic separation is also a good choice[14].

To improve HPLC-MS/MS for fast separation and determination of tobacco-specific nitrosamines in cigarette smoke, a UPLC-MS/MS method has been developed and validated using four isotopically labeled internal standards. A mixed-mode cation-exchange solid phase extraction (SPE) column was used for sample purification. During analysis, chromatographic baseline separation of four tobacco-specific nitrosamines was achieved by using an UPLC column, further enhancing the method's accuracy and robustness. This new method has been successfully applied to tobacco-specific nitrosamines analyses for 2003 Kentucky reference cigarette (KY2R4F) and some Chinese brand cigarettes.

EXPERIMENTAL

Tobacco-specific nitrosamines standards (N-nitrosonornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone,

N-nitrosoanatabine and N-nitrosoanabasine) and four internal standards (N-nitrosonornicotine-d4, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-d4, N-nitrosoanatabine-d4 and N-nitrosoanabasine-d4) were obtained from Toronto Research Chemicals Inc. (Toronto, Canada). All standards and internal standards were dissolved in acetonitrile, diluted to intermediate stock solutions at 1 µg/mL and stored at -20°. Intermediate stock solutions were used to prepare calibration standard solutions. Commercially available solid phase extraction columns (PCX columns) were obtained from Agela Company (Beijing, China). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). 2003 Kentucky reference cigarette 2R4F was purchased from Kentucky Tobacco Research and Development Center, University of Kentucky (USA). Popular Chinese cigarettes were collected in Chinese market.

Sample preparation: Cigarettes and cambridge filter pads (CFPs, Whatman, UK) to be used for this study were conditioned at 22 ± 1 °C and a humidity of 60 ± 2 % for at least 48 h. All cigarettes were smoked in a puff volume of 35 mL with 2 s puff duration in every 60 s by using a 20-port Borgwaldt RM200 smoking machine (Borgwaldt, Germany). The smoke particulate matter from 20 cigarettes was collected in one cambridge filter pads with a diameter of 92 mm. The cambridge filter pads was then transferred into a 250 mL erlenmeyer flask and 50 µL (1 µg/mL) of internal standard mixtures and 40 mL of the extraction solution (100 mM ammonium acetate) were added. Finally, the target analytes captured in cambridge filter pads were ultrasonically extracted into solution phase for 40 min under room temperature. After this, the pH value of extraction solution was adjusted to 1.5 with hydrochloric acid and 2 mL was taken to be loaded onto the solid phase extraction column.

In our study, a mixed-mode cation-exchange PCX cartridge (3 mL, 60 mg) was employed to clean up sample matrix. Prior to solid phase extraction procedure, conditioning of the cartridge was conducted with 3 mL of methanol and followed by 3 mL of water. After loading of 2 mL sample, the cartridge was then washed with 3 mL methanol and eluted with 3 mL ammonia/methanol (5 %, V/V). Finally, the eluent was transferred into an autosampler vial by using a syringe filter and analyzed by UPLC-MS/MS.

UPLC-MS/MS analysis: Sample analysis was carried out using an Agilent 1200 liquid chromatograph coupled with Agilent 6410 triple quadruple mass spectrometer (Agilent Technologies, USA). Completely chromatographic baseline separation of tobacco-specific nitrosamines from smoke extract was achieved using reversed phase UPLC with an Agilent XDB-C_{18} column (50 mm × 4.6 mm i.d. 1.8 µm) at 50 °C (Fig. 1). The mobile phase was 30 % methanol, 70 % water and the flow rate was 0.4 mL/min. The total run time for each sample was 10 min. Ionization of target analytes was carried out using the electrospray ionization (ESI) source in positive ion mode. The ESI source parameters were set as follows: ion spray voltage at 3000 v, cone voltage at 35 V, source temperature at 350 °C, aux gas flow rate at 11 L/min, and collision gas pressure at 2.45 × 10^{-5} Torr. Compound-dependent parameters were optimized using flow injection analysis at a constant flow rate of 10 µL/min. Analytes and internal standards were monitored by different precursor and product ion transition pairs (Table-1) under multiple reaction monitoring (MRM) mode. All data for samples were processed by using the Agilent chemstation software version B.01.00.

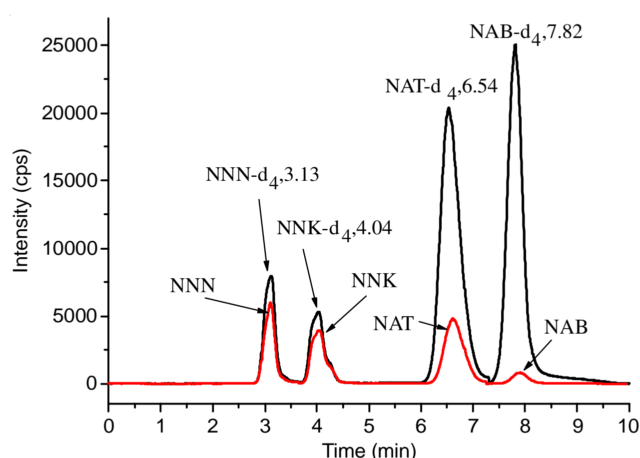

Fig. 1. Typical chromatographic separations of tobacco-specific nitrosamines (TSNAs) and isotopic internal standards

RESULTS AND DISCUSSION

Sample preparation: In previous study[7], it was demonstrated that 10-30 mL of 100 mM ammonium acetate solution could make an adequate and selective extraction of tobacco-specific nitrosamines from the cigarettes and cambridge filter

TABLE-1
MULTIPLE REACTION MONITORING ANALYSIS OF FOUR TOBACCO-SPECIFIC NITROSAMINES AND ITS INTERNAL STANDARDS

Analyte	m.w.	Precursor ions	Product ions	CE (eV)[a]	FE (V)[b]
NNN	177	178	148[c]	5	50
		178	120[d]	12	50
NNK	207	208	178[d]	4	60
		208	122[c]	8	60
NAT	189	190	160[c]	4	60
		190	106[d]	10	60
NAB	191	192	162[c]	4	60
		192	133[d]	16	60
NNN-d4	181	182	152	5	50
NNK-d4	211	212	126	4	60
NAT-d4	193	194	164	4	60
NAB-d4	195	196	166	4	60

[a]CE, Collision energy; [b]FE, Fragment energy; [c]Quantitation ion; [d]Confirmation ion; NNN, N-Nitrosonornicotine; NNK, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, N-Nitrosoanatabine; NAB, N-Nitrosoanabasine

pads. Considering some cigarettes may produce more tobacco-specific nitrosamines, 40 mL was selected for extraction in our study. To optimize the extraction time of tobacco-specific nitrosamines, a series of reference cigarettes were smoked and the cigarettes and cambridge filter pads were ultrasonically extracted with ammonium acetate for 20, 40, 60 and 120 min, respectively. The results indicated that higher extraction efficiency was obtained for the extraction time of 40 min. Consequently, 40 min was finally selected as the best one.

In order to diminish the matrix effect, further clean-up procedure of the smoke extraction was performed using solid phase extraction technology. In this paper, a series of commercially available solid phase extraction columns: basic aluminum cartridge, C_{18} cartridge, and PCX cartridge (60 mg/3 mL, Agela company, China) were evaluated to obtain optimal separation and recovery. It was found that the PCX cartridge had the best selectivity and recovery for the separation of target tobacco-specific nitrosamines. The possible reason for this was due to the new material of mixed-mode cation-exchange PCX cartridges. In general, sorbents packed in solid phase extraction cartridges mainly include non-polar phase, ion-exchange phase and polymeric phase. Benefitted from the mixed-mode retention mechanism of PCX material, the protonated tobacco-specific nitrosamines were more easily to be reserved than polar or non-polar substances. Considering both removal of unwanted components and retaining as much of the tobacco-specific nitrosamines as possible on PCX cartridge during washing process, a series of preliminary experiments were carried out by evaluating the recovery of tobacco-specific nitrosamines. The final optimal solid phase extraction conditions were as follows: the pH value of loading solution was adjusted to 1.5, washing with 3 mL methanol, eluting with 3 mL 5 % ammonia/methanol solution (v/v).

Optimization of chromatographic separation conditions: Two types of LC columns, including Agilent Zorbax C_{18} (150 mm × 4.6 mm i.d., 5 μm) and Agilent XDB-C_{18} (50 mm × 4.6 mm i.d., 1.8 μm) columns, were experimented to obtain the maximum resolution and optimal peak shape of tobacco-specific nitrosamines. After careful evaluation of these two columns, it is observed that a good separation of tobacco-specific nitrosamines and individual internal standard could be achieved on Agilent XDB-C_{18} column using an isocratic elution and the mobile phase consisting of water and methanol. For Agilent Zorbax C_{18} column, the four tobacco-specific nitrosamines were separated with very low resolution and relatively long analysis time was taken on it. Results showed that UPLC analysis based on the use of columns, packed with stationary phases of particle size (< 2 μm) smaller than conventional HPLC, can be used as a powerful tool for the separation of tobacco-specific nitrosamines. It led to higher resolution and sensitivity, and shorter analysis time. During the optimization of mobile phase, it was found that the presence of acetic acid could strongly influence the retention character of tobacco-specific nitrosamines. The chromatographic baseline separation of tobacco-specific nitrosamines was achieved using an isocratic elution with the mobile phase of 70 % water and 30 % methanol. Actually, a faster LC separation could be obtained using a gradient with mobile phase methanol increased from 25 % at 0 min to 35 % at 2 min. However, considering the effect of solvent variations on analyte ionization, a gradient was ultimately abandoned. In addition, the shoulder effect and peak splitting previously reported by Jasson *et al.*[15] were also observed in present UPLC separation conditions. However, these phenomena which are temperature-dependant could be avoided at the column temperature of 50 °C. Fig. 1 shows the chromatogram of tobacco-specific nitrosamines and internal standards in KY2R4F cigarette mainstream smoke sample.

Method performance: The calibration curves were prepared in the range of 0.8-100 ng/mL for tobacco-specific nitrosamines with addition of 50 ng individual internal standard. Excellent linearity was obtained with the correlation coefficient values (r) between 0.9988 and 0.9995. The limit of quantification (S/N > 10) for N-nitrosonornicotine, 4-(methyl-nitro-samino)-1-(3-pyridyl)-1-butanone, N-nitrosoanatabine, N-nitrosoanabasine were 0.23, 0.29, 0.25 and 0.35 ng/mL, respectively.

Method accuracy was evaluated by comparing the determination of KY2R4F reference cigarette and some Chinese brand cigarettes with standard GC-TEA method. Table-2 shows the different results of KY2R4F reference cigarette determined both by UPLC-MS/MS and GC-TEA. By comparison conventional true value[7], it is found that satisfactory results were obtained by using UPLC-MS/MS method. In recovery experiment, tobacco-specific nitrosamines were added at concentration of 5 ng/mL, the recovery were obtained from 89 to 91 % with the RSD (n = 5) ranging from 2.3 to 7.8 %, respectively.

Conclusion

This paper shows that UPLC-MS/MS is a rapid, reliable, selective and sensitive technique for the quantitative determination of trace tobacco-specific nitrosamines in cigarette smoke. With a chromatographic run time of 10 min, four tobacco-specific nitrosamines were completely separated and determined, with a prior solid phase extraction technology. Chromatographic baseline separation and the use of solid phase extraction can effectively diminish interferences of matrix compounds. All of these lead to higher selectivity and sensitivity in quantitative analysis of tobacco-specific nitrosamines.

TABLE-2
COMPARISON OF RESULTS FOR TOBACCO-SPECIFIC NITROSAMINES IN KY2R4F REFERENCE CIGARERTTE SMOKE

Compounds	UPLC-MS/MS[(a)]	GC-TEA[(b)]	LC-MS/MS[(c)]	Conventional true value[(c)]
NNN (ng/cig)	138	149	152	146
NNK (ng/cig)	114	129	133	141
NAT (ng/cig)	123	133	112	143
NAB (ng/cig)	15.2	16.1	11.8	16.6

[(a)]This study obtained by UPLC-MS/MS; [(b)]This study using GC-TEA; [(c)]Data reported by Wu *et al.*[7]; NNN, N-Nitrosonornicotine; NNK, 4-(Methylnitro-samino)-1-(3-pyridyl)-1-butanone; NAT, N-Nitrosoanatabine; NAB, N-Nitrosoanabasine

This method is more suitable for identification and determination of tobacco-specific nitrosamines in low levels, including Chinese flue-cured cigarettes.

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