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Metabolism Study on Tobacco-specific N-Nitrosamines in Rabbit by Solid-phase Extraction and Liquid Chromatography-electrospray Ionization-mass Spectrometry Method

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Abstract: Metabolism of four tobacco-specific N-nitrosamines (TSNAs), N′-nitrosonornicotine (NNN), N′-nitrosoanatabine (NAT), N′-nitrosoanabasine (NAB) and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was studied by solid-phase extraction (SPE) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). SPE and LC-ESI-MS method was evaluated to be rapid, simple, sensitive and selective for analysis of TSNAs in rabbit serum. The doses of TSNAs administrated were 4.666 μg/kg and 11.665 μg/kg according to different levels in cigarettes. Metabolic curves of four TSNAs and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNAL), the metabolite of NNK, were obtained.

Keywords: Tobacco-specific N-nitrosamines, metabolism, liquid chromatography-electrospray interface-mass spectrometry.

Tobacco-specific nitrosamines (TSNAs) are a group of carcinogens found only in tobacco products. They are formed by nitrosation of nicotine and related tobacco alkaloids [1]. Seven tobacco-specific nitrosamines have been identified in tobacco products [2, 3], and almost all studies to date have been carried out with NNN, NNK, NAB, NAT and NNAL. In this paper, metabolism of NNN, NNK, NAB and NAT in rabbit was studied by solid-phase extraction (SPE) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). SPE and LC-ESI-MS method was evaluated to be rapid, simple, sensitive and selective for analysis of TSNAs in rabbit serum. The metabolic curves of four TSNAs and NNAL, the metabolite of NNK, were obtained. This work is important for further research on carcinogenicity of TSNAs.

Experimental

Japanese male white (JW) domestic rabbits, 4 months old, body weight (BW) between 2.2 and 2.5 kg were offered by the Tongli Experimental Animal Center of Beijing. Before administration of TSNAs, all rabbits were unfed for 12 h. Blank blood was collected just before TSNAs administration. After that, rabbit were administrated with

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a mixture of four TSNAs: NNN, NNK, NAT and NAB dosed in accordance with their weight (4.666 μg/kg and 11.665 μg/kg according to different levels in cigarettes). We put rabbits in the metabolism cages to collect 2 mL blood each time from rabbit auricular vein and recorded the elapsed time. About 0.5 mL serum sample was obtained by ultracentrifugation for 10 min at 2200 rpm and 15°C and frozen at -70°C before analysis.

Serum samples were thawed and transferred to a C18 SPE column (Yunquan, Beijing, PRC), then preconditioned with 1 mL methanol and 1 mL water. The column was rinsed with 0.5 mL water then the TSNAs were eluted with 2 mL methanol. The extract was dried under nitrogen and diluted with 100 μL methanol before analysis on LC-ESI-MS.

LC-MS experiments were performed on an Agilent 1100 HPLC system coupled to an Agilent XCT trap mass spectrometry equipped with an electrospray ion source (Agilent Technologies, CA, USA). A 150×2.1 mm, i.d. 5 μm Lumex (Lumex, Russia) Kromasil C18 column preceded by a 4×2.1 mm Agilent guard column was utilized for separation and maintained at 20 ℃. The mobile phase A was 10 mmol/L ammonium formate at pH 4 in water–methanol (80:20, v/v) and B was methanol. The gradient was: t=0 min, 0% B; t=6 min, 20% B; t=15 min, 0% B, and the interval between injections was 20 min. The flow-rate was 0.2 mL/min, and the injection volume was 5 μL. LC-MS was operated in the positive ion mode and the experimental conditions were as follows: nitrogen as the nebulising gas at 40 psi, the drying gas was heated to 300 ℃ at a flow-rate of 8 L/min, the capillary voltage was set at 3.0 kV and scan range was from 50 *m/z* to 250 *m/z*.

Results and Discussion

HPLC performance regarding retention time, response and peak shape was tested at different pHs: 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and different gradients. The best performance of separation for each TSNA was achieved at pH 4.0. Optimization conditions were described in experimental section and the chromatogram of five TSNAs was showed in **Figure 1**. From the chromatogram, NNN was divided into two peaks. The fragmentation pattern of both NNN peaks was exactly the same, which means that both peaks, most likely, came from the same compound. The split up of the signal for NNN into two peaks was probably due to the basicity of NNN and the formation of two structural isomers after the protonation of NNN[4].

This SPE and LC-ESI-MS method was evaluated for the analysis of TSNAs in rabbit serum. Detection limits, linear ranges, linearities and repeatabilities for the determination of these five analytes were listed in **Table 1**. The recovery in serum was 88.3%-95.6% for addition of 0.2 μg/mL standards and 85.5%-101.1% for 0.01 μg/mL.

Metabolic time curves of four TSNAs under different concentrations of TSNAs administrated were obtained as showed in **Figure 2**, showing that serum concentrations of NNN, NNK, NAT and NAB were quickly decreased in 30 minutes. NAT almost consumed out by metabolism in 15 minutes and NNK needs half an hour to finish its metabolic procedure. NAT and NNN performed less metabolic activation and their metabolic procedures need about 90 minutes. As we can see, when the initial concen-

Figure 1 Total ion chromatogram of NNN, *iso*-NNAL, NNK, NAT, NAB

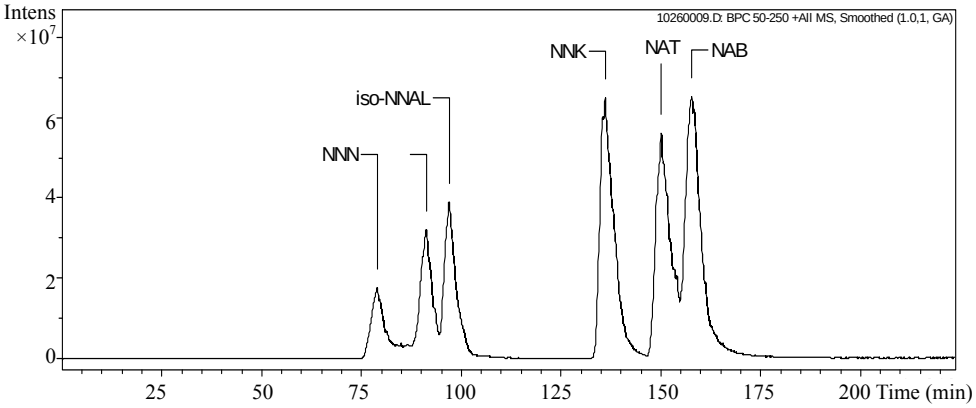


Table 1 Detection limits, linear ranges, linearities and repeatabilities (n=5) for determination of five TSNAs

Analytes	Detection limit (ng/mL)	Linear range (μg/mL)	R^2	Intra-day RSD (%)	Inter-day RSD (%)
NNN	0.5	0.0050-1.5	0.999	4.8	7.2
iso-NNAL	0.5	0.0050-1.4	0.998	4.5	6.9
NNK	0.5	0.0050-1.6	0.998	1.0	2.2
NAT	0.1	0.0010-1.2	0.999	0.48	1.3
NAB	0.1	0.0010-1.1	0.998	0.31	0.91

trations of TSNAs changed, the metabolic time curves presented similar shapes but different slopes, showing that the metabolic rates were related to the initial concentrations.

NNK, as one of the most carcinogenic of TSNAs, was rapidly converted to NNAL metabolically in humans, rodents and primates[1]. The metabolite NNAL was detected in rabbit serum by this method and its metabolic curve was indicated in **Figure 3**. About half of the NNK converted to NNAL under the two initial concentrations as can be seen from the curves. NNAL, like NNK, is a potent carcinogen after further metabolism. These results are important for further study on exact metabolic pathways of TSNAs, which is in progress in our lab.

Conclusion

This work developed a rapid, simple, sensitive and selective SPE and LC-ESI-MS method for analysis of TSNAs in rabbit serum. This method was successfully applied to study the metabolism of TSNAs in rabbit. Metabolic time curves of NNN, NNK, NAT, NAB and NNAL, the metabolite of NNK were obtained, which proved the first metabolic step of NNK and accumulated useful data for further study on exact pathways and carcinogenicity of TSNAs.

Figure 2 Metabolic time curves of NNN, NNK, NAT and NAB. The initial concentrations of four TSNAs were a) 11.665 μg/kg, and b) 4.666 μg/kg.

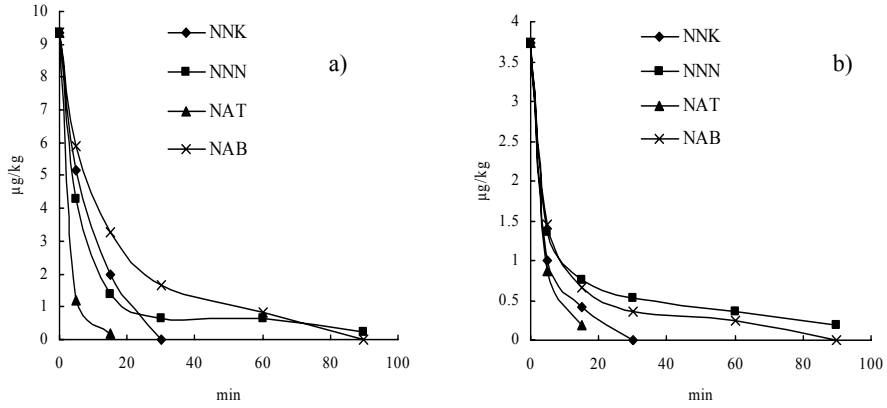
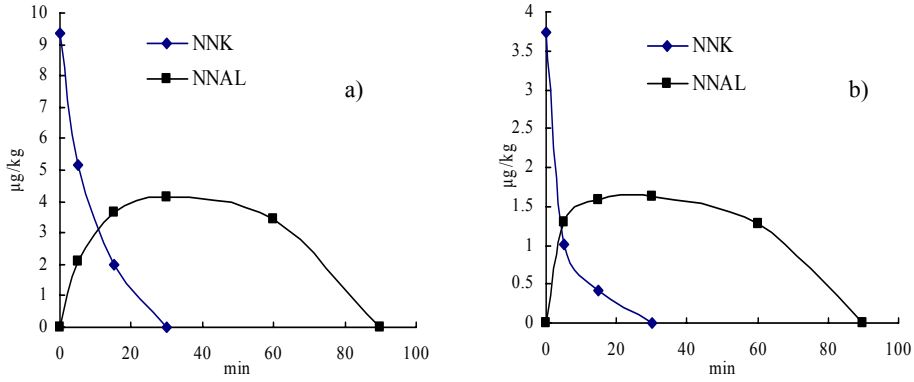


Figure 3 Metabolic time curves of NNK and its metabolite, NNAL. The initial concentrations of four TSNAs were a) 11.665 μg/kg, and b) 4.666 μg/kg.



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