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Analysis of N′-nitrosonornicotine and its metabolites in rabbit blood with liquid chromatography/tandem mass spectrometric method

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ABSTRACT

N′-nitrosonornicotine (NNN) is a strong carcinogen. The metabolic study of NNN in vivo will help us to further understand it, however, trace detection in complex matrices requires highly sensitive detection methods. After the chromatographic conditions and mass spectrometric conditions had been optimized and confirmed, a method for determining NNN and its metabolites in rabbit blood by high-performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) was established. The results showed that precisions (R.S.Ds) were between 0.5% and 8.62%, the recoveries ranged from 80% to 111%. Linearity was observed for all compounds with detection limits ranging from 0.039 ng mL^{-1} to 0.217 ng mL^{-1}. Metabolic curves and pharmacokinetic parameters were obtained for NNN and its metabolites. The elimination half-life of NNN was 30 min and the main metabolite of NNN was 4-hydroxy-4-(3-pyridyl)-butyric acid (hydroxy acid) and the major metabolic pathway was 5′-hydroxylation and subsequent secondary metabolite formation.

1. Introduction

N′-nitrosonornicotine (NNN) is a tobacco-specific nitrosamine (TSNA) formed by nitrosation of the tobacco alkaloid and metabolite nornicotine [1]. NNN can cause tumors of the esophagus and nasal mucosa in rats, respiratory tract in hamsters, lung in mice [2] and is likely to play an important role as a cause of esophageal cancer in smokers and oral cavity tumors in people who use smokeless tobacco products [3]. NNN is now considered as a leading class carcinogen by the IARC [4] and metabolic activation is considered to be a prerequisite for its mutagenicity. It has three known metabolic pathways in rodents [2]: pyridine-*N*-oxidation produces N′-nitrosonornicotine-1-*N*-oxide (NNN-*N*-oxide); denitrosation and oxidation yield norcotinine and hydroxylation occurs α- to the N-nitroso group to give either 2′-hydroxyNNN or 5′-hydroxyNNN [5]. The 3′-hydroxyNNN and 4′-hydroxyNNN isomers of hydroxylation are also known as minor metabolites in rodents. Myosmine, 1-(3-pyridyl)-1,4-butanediol (PBD), 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), and 4-oxo-4-(3-pyridyl)-butyric acid (keto acid) are the products subsequent oxidation of 2′-hydroxyNNN oxidation while 5′-hydroxyNNN is further metabolized to 4-hydroxy-4-(3-pyridyl)-butyric acid (hydroxy acid), as shown in Fig. 1.

Upadhyaya [5] pointed out that the hydroxy acid and norcotinine were the major metabolites of NNN in Patas monkey's blood. In rodent's urine, the major metabolites of NNN were the keto acid and hydroxy acid. Since NNN is a strong carcinogen, it is desirable to understand its metabolic characteristics. However, some metabolites of NNN are very low in concentration and for this reason a sensitive and rapid analytical method is needed.

Many methods have been developed to analyze NNN and its metabolites due to the risk they pose to human health. High performance liquid chromatography combined with thermal energy analysis detection (HPLC-TEA) and gas chromatography combined with thermal energy analysis detection (GC-TEA) have been frequently cited for this analysis [5–7] but both lack the specificity of analysis that is offered by mass spectrometry. Compared with HPLC and GC, HPLC–MS/MS has advantages including simplified pre-treatment steps, low detection limits and short analysis times. HPLC–MS/MS has previously been applied to analyzing TSNAs in tobacco products [8] and rabbit's blood [9,10], total NNN in smokers' urine [11–13] and NNK metabolites in A/J mouse urine [14]. To our knowledge, there have been no reports describing the analysis of metabolites of NNN using HPLC–MS/MS.

In this study, a HPLC–MS/MS method was developed and applied to determining NNN and its metabolites in rabbit blood. The metabolic curves and pharmacokinetic parameters for NNN and its metabolites were also obtained.

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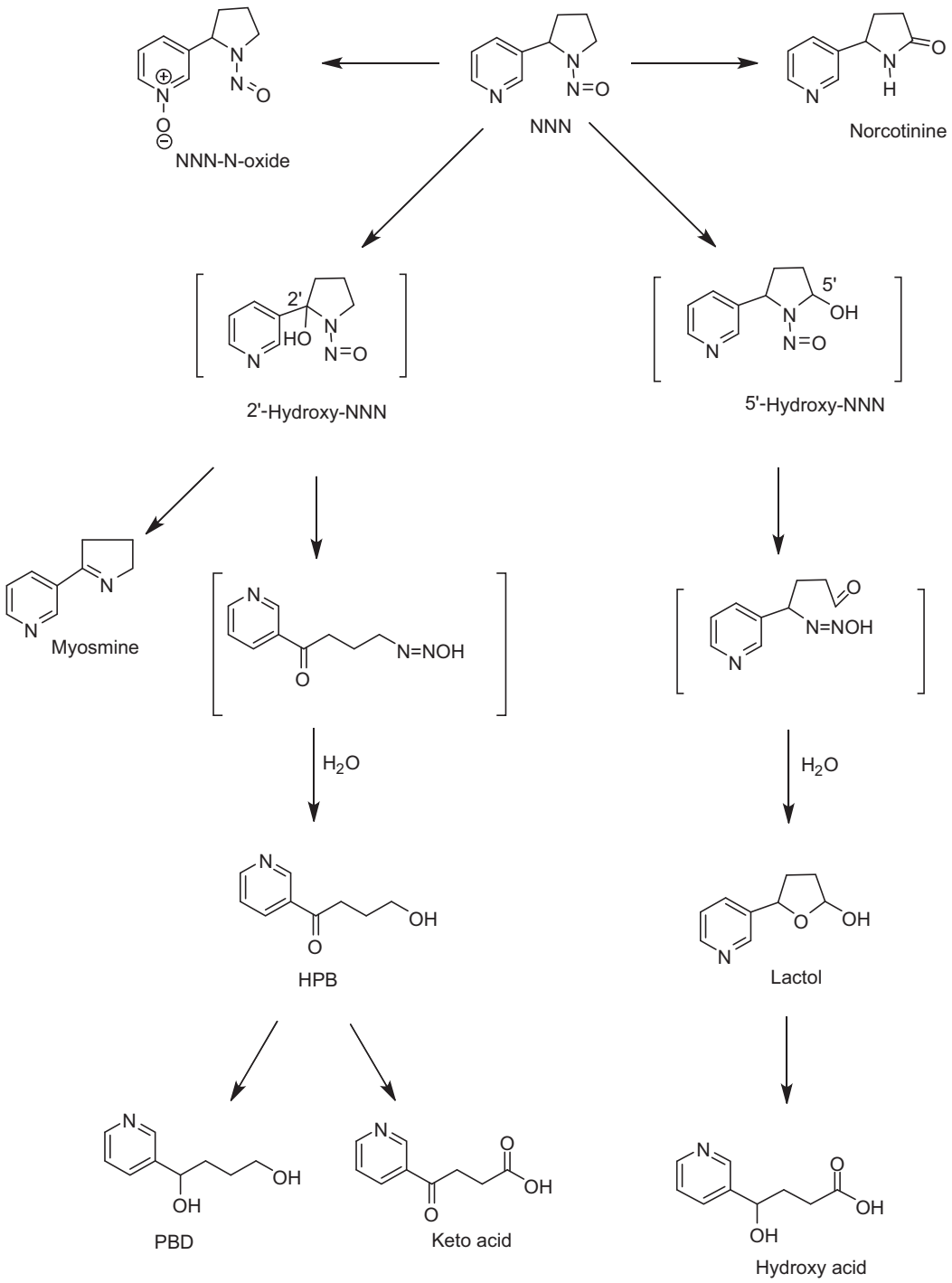

Fig. 1. Metabolites of NNN based on studies in rodents.

2. Experimental

2.1. Chemicals

N′-nitrosonornicotine (NNN), NNN-*N*-oxide, myosmine, 4-oxo-4-(3-pyridyl)-butyric acid (keto acid), norcotinine, 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), 1-(3-pyridyl)-1,4-butanediol (PBD), and 4-hydroxy-4-(3-pyridyl)-butyric acid (hydroxy acid) and NNN-d_4 were purchased from Toronto Research Chemicals Inc. (North York, Canada).

The purified water was prepared by use of a Milli-Q system (Millipore, USA). Formic acid, acetic acid, ammonium formate and ammonium acetate were purchased from Tedia Company Inc. (HPLC grade, USA). Methanol and acetonitrile were provided by J.T. Baker Company Inc. (HPLC grade, USA). Dipotassium EDTA was purchased from Kemio Company Inc. (>99.0%, China).

2.2. Standard solution preparation

All of the standard samples were prepared as 1 mg mL^{-1} stock solutions in methanol and were stored at −40 °C. A series of working standard solutions were prepared by diluting the stock solutions with different amount of 90% acetonitrile and 10% water solution

containing 10 mM ammonium formate (the mobile phase of this experiment) to give concentrations of 1000 ng mL^{-1} for NNN and 100 ng mL^{-1} for each metabolite, which were identified as standard solutions. Plasma-based calibration solutions were prepared by spiking substance free plasma at concentrations of 2, 5, 10, 20, 50, 100, 200, 400, and 800 ng mL^{-1} for NNN and 0.2, 0.5, 1, 2, 5, 10, 20, 40, and 80 ng mL^{-1} for each of the metabolites of NNN. Three other standard solutions were also prepared, independently, as quality control samples, and were diluted to yield low (5 ng mL^{-1} for NNN and 0.5 ng mL^{-1} for metabolites), medium (10 ng mL^{-1} for NNN and 1 ng mL^{-1} for metabolites), and high (40 ng mL^{-1} for NNN and 4 ng mL^{-1} for metabolites) controls in rabbit plasma. The final concentration of NNN-d_4 (IS) in each standard solution was 2 ng mL^{-1}.

2.3. Blood sample preparation

Japanese domestic rabbits, body weight between 2.5 and 3 kg, were bought form Yu-Hua Rabbit Breeding Center (Zhengzhou, China). They were all males and 4 months old. Rabbits were housed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals at Animal Breeding Center (Zhengzhou, China). All rabbits were fasted for 12 h prior to injection of NNN (0.8 mg kg^{-1}) which was dissolved in 0.7 mL 9% physiological saline through the auricular vein. A 0.5 mL blood sample was obtained from the opposite auricular vein at 2, 5, 10, 30, 60, 90, 120, 240, and 360 min after injection and introduced to a 1.5 mL Eppendorf tube which contained 100 μL of saturated dipotassium EDTA solution. The plasma was obtained by ultracentrifugation for 15 min at 10,000 rpm and 4 °C and frozen at −80 °C until analysis.

A 0.3 mL portion of the plasma was transferred to another 1.5 mL Eppendorf tube by pipette and mixed with 60 μL of IS solution containing 2 ng IS. In order to precipitate proteins, methanol was added to give a final volume of 1 mL. The treated samples were placed in a refrigerator (about 4 °C) for 30 min, and then ultracentrifugated for 3 min at 2000 rpm. The supernatant liquid was filtered through a 0.22 μm Nylon filter (Agilent, USA) and transferred to an autosampler vial prior to analysis.

2.4. LC–MS/MS analysis

Liquid chromatography–mass Spectrometry was performed with an Agilent Technologies (CA, USA) 1200 series HPLC system with a vacuum degasser (G1322A), a quaternary gradient pump (G1311A), an autosampler (G1329A) and a column compartment (G1316A) coupled to an API 4000 operating with a TurboIonSpray source (Applied Biosystem, Ontario, Canada).

Compounds were separated on a Waters Atlantis HILIC Silica column (100 mm × 3.0 mm i.d. 3 μm particle, USA) at the flow rate of 500 μL min^{-1}. The optimized mobile phase used a linear gradient of A (water solution containing 10 mM ammonium formate) and B (acetonitrile): 10:90 A/B (v/v) from 0 to 2 min; 25:75 A/B from 2 to 7 min; 10:90 A/B from 7 to 7.5 min, holding this composition until 10 min. The injection volume was 2 μL and the column temperature was maintained at 26 °C.

The mass spectrometer was operated in positive ion ESI mode with an ion spray voltage of 5500 V. Turbospray settings were as follows: nebulizer and curtain gas was 20 psi, collision-assisted dissociation (CAD) at 8 psi, turbo gas at 8 L min^{-1}, and the source heater probe temperature at 600 °C. Quantitative analysis of NNN and its metabolites used multiple reactions monitoring (MRM) mode, which included precursor ions, product ions, declustering potential (DP) and collision energy (CE) as listed in Table 1.

Table 1
Operation parameters of simultaneous analysis of NNN and its metabolites by LC–MS/MS in MRM mode.

Analyte	Q1 (m/z)	Q3 (m/z)	DP[a] (v)	CE[b] (v)
NNN	178.2	148.2	50	15
Keto acid	180.1	134.1	46	29
Hydroxy acid	182.1	164.1	46	23
Norcotinine	163.1	80.1	59	35
PBD	168.1	108	46	47
NNN-*N*-oxide	194.2	147.2	45	23
Myosmine	147.2	105.1	50	33
HPB	166.1	106.1	50	24
NNN-d_4	182.1	152.1	50	17

[a] Declustering potential.
[b] Collision energy.

3. Results and discussion

3.1. Method development

Many variables affect separation performance and signal intensities, including the column and mobile phase. To achieve maximum sensitivity and get better peak shapes, we evaluated different solvent systems, including methanol–water and acetonitrile–water both containing ammonium acetate or ammonium formate. Under the starting condition of 10:90 A (water containing 10 mM ammonium formate) and B (acetonitrile) (v/v), the separation of all of the compounds with well-defined peak shapes and maximum sensitivity was observed, as shown in Fig. 2.

The fragmentation conditions used for each compound are described in Table 1. One major advantage of MS–MS for trace analysis is its confirmatory power for analytes by the way of characteristic product to precursor ion transitions. MRM conditions were optimized by infusions of standard solutions of each analyte.

3.2. Method validation

3.2.1. Calibration curve and limit of detection

The linearity was determined using several samples (2–800 ng mL^{-1} for NNN and 0.2–80 ng mL^{-1} for its metabolites) and calibration curves were obtained with correlation coefficient values (r) between 0.9991 and 1.0000, as shown in Table 2. The limit of detection (LOD) was defined by peak heights, 3 times higher than the maximum baseline height of blank, which is also listed in Table 2.

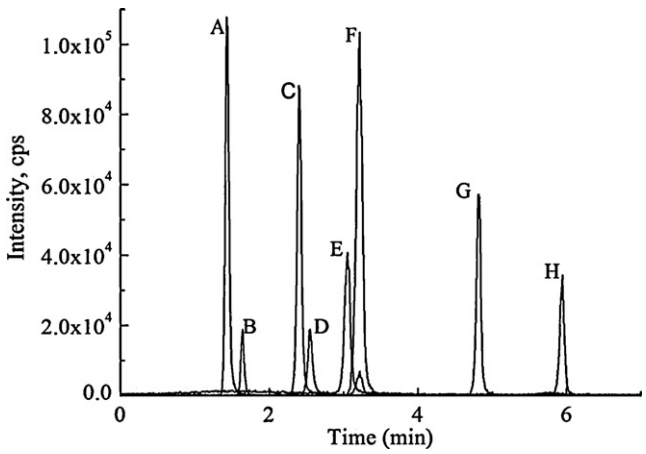

Fig. 2. LC–MS/MS chromatogram of reference compounds using MRM (multiple reaction monitor) mode with acetonitrile–water (containing 10 mM ammonium formate) as the mobile phase.

Table 2
Calibration curve and LOD of the NNN and its metabolites.

Analyte	Linear regression equation	r	LOD (ng mL^{-1})
NNN	$Y = 0.344X + 0.0697$	1.0000	0.039
Keto acid	$Y = 0.294X - 0.170$	0.9994	0.053
Hydroxy acid	$Y = 0.114X - 0.0478$	0.9991	0.058
Norcotinine	$Y = 0.0951X - 0.0456$	0.9991	0.217
PBD	$Y = 0.249X - 0.053$	0.9997	0.097
NNN-*N*-oxide	$Y = 0.624X - 0.174$	0.9995	0.049
Myosmine	$Y = 0.0911X - 0.0204$	0.9998	0.114
HPB	$Y = 0.278X - 0.0624$	0.9997	0.066

Table 3
Recoveries and R.S.Ds. of NNN and its metabolites in rabbits' plasma.

Analyte	Concentrations of analytes (ng mL^{-1})	Mean recovery (%) ($n = 5$)	R.S.D (%) ($n = 5$)
NNN	5	106	3.26
	10	104	2.00
	40	92.3	0.500
Keto acid	0.5	111	1.73
	1	111	3.97
	4	94.5	2.02
Hydroxy acid	0.5	108	4.32
	1	81.6	5.22
	4	105	2.71
Norcotinine	0.5	85.0	5.35
	1	108	8.62
	4	91.0	3.08
PBD	0.5	93.0	5.16
	1	98.4	6.04
	4	92.8	3.57
NNN-*N*-oxide	0.5	95.0	3.09
	1	106	4.91
	4	97.3	2.63
Myosmine	0.5	87.0	4.67
	1	80.0	4.00
	4	94.8	2.03
HPB	0.5	108	2.59
	1	104	4.75
	4	95.0	2.60

3.2.2. Accuracy and recovery

The repeatability of analysis and recovery of NNN and its metabolites from blank plasma were determined at three different concentrations (5, 10, and 40 ng mL^{-1} for NNN; 0.5, 1, and 4 ng mL^{-1} for other analytes). Accuracies at low, medium and high levels of NNN and its metabolites were determined with spiked rabbit blood samples. Each level was analyzed 5 times. Recovery is corrected for matrix effects and is obtained by comparing the peak areas obtained from extracted, spiked plasma samples (at three levels) with similar plasma samples that were extracted and the extract then identically spiked with analytes of interest. The R.S.D and mean recovery ($n = 5$) are listed in Table 3. The recoveries were between 80% and 111%, R.S.Ds were 0.5–8.62%.

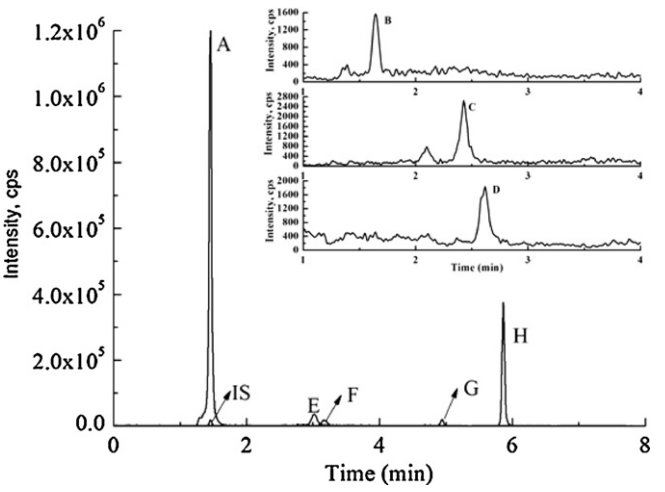

Fig. 3. LC–MS/MS chromatogram of NNN and its metabolites in male Japanese white domestic rabbits' plasma using MRM (multiple reaction monitor) mode.

Table 4
Mean pharmacokinetic parameters ($n = 5$) for NNN and metabolites in rabbits' blood.

	$t_{1/2}$ (min)	AUC (ng mL^{-1} h)	t_{max} (min)
NNN	18.5	3884.7	2
Keto acid	10.5	481.9	9
Hydroxy acid	55.2	10,457.5	17
NNN-*N*-oxide	10.6	187.4	2
Norcotinine	69.5	1416.4	11
HPB	122.3	36.2	3
PBD	86.4	183.6	6
Myosmine	49.9	30.4	3

3.3. Detection of metabolites in rabbit blood

A typical chromatogram is shown in Fig. 3. Metabolic curves of NNN and its metabolites were constructed by plotting the mean concentrations ($n = 5$) as ordinate and the times elapsed following administration as abscissa (Fig. 4).

The plasma concentration versus time data for NNN and its metabolites in experiments were analyzed by noncompartmental analysis [15]. The slope of the terminal phase of the plasma concentration rate versus time curve was determined by fitting the data to a monoexponential equation. The terminal rate constant, λ, was determined from the slope. For both NNN and its metabolites, the area under the plasma concentration–time curves from time 0 to time t [AUC (0–t)] was determined by the linear trapezoidal rule up to the last measured concentration. The AUC (t–∞) was determined by dividing the last measured concentration by the terminal rate constant. The AUC (0–∞) was the sum of the two partial AUCs. The elimination half-life ($t_{1/2}$) was calculated as 0.693 divided by λ.

Table 5
Total concentrations of metabolites from each metabolic pathway in different times.

Time (min)	2′-Hydroxylation	5′-Hydroxylation	Formation of norcotinine	Pyridine-*N*-oxidation
2	4.23	4.33	2.97	3.35
5	3.94	16.4	3.33	1.57
10	1.43	33.0	3.74	0.71
30	1.69	62.3	3.09	1.12
60	2.08	55.2	3.23	1.60
90	2.94	56.7	5.29	2.11
120	3.87	62.3	6.28	2.50
240	4.50	81.1	6.70	1.47
360	0.19	82.0	9.52	NS[a]

[a] No signal.

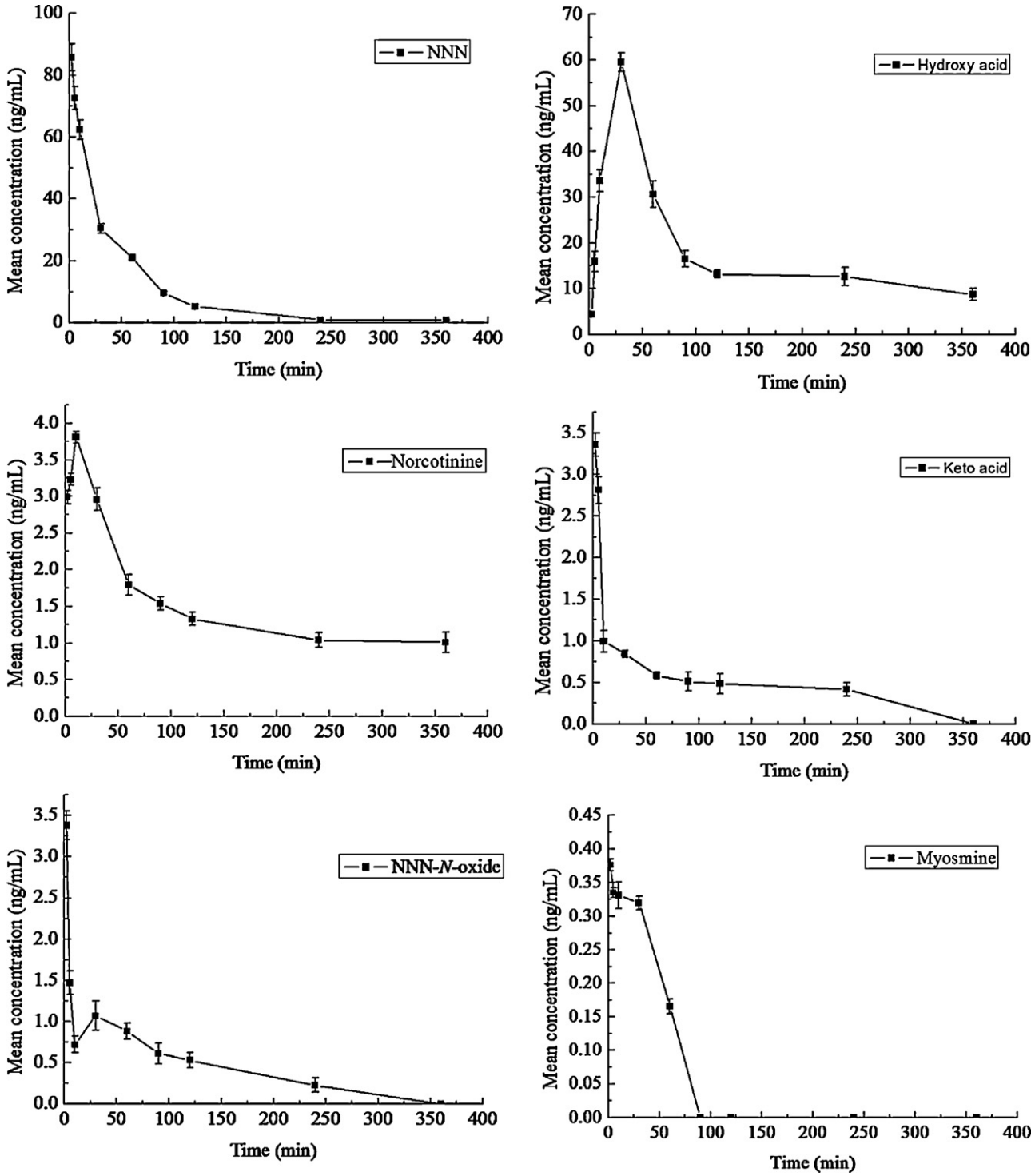

Fig. 4. Mean plasma concentration–time profile ($n = 5$) for NNN and its metabolites in the plasma of Male Japanese white domestic rabbits.

Analysis of pharmacokinetic parameters (Fig. 4, Table 4) demonstrated that NNN was rapidly eliminated from the body in an apparent monoexponential manner with an elimination half-life of 18.5 min. The major metabolite in rabbit plasma was the hydroxy acid, as indicated by the AUC. NNN was rapidly metabolized to the hydroxy acid, with its maximal plasma concentration (t_{max}) occurring at 17 min. The hydroxy acid had an apparent elimination half-life of 55.2 min. The formation of norcotinine, keto acid, NNN-*N*-oxide, PBD, HPB, and myosmine was also very fast with the t_{max}s within 11 min of administration. HPB was very slowly eliminated, once formed, with an elimination half-life of 122.3 min. The amounts of PBD, HPB, and myosmine were quantitatively less significant than any other metabolites, especially myosmine, which could not be detected after 90 min. Although the quantity of HPB was very small, it could still be quantified at 360 min. Mechanistically, this may suggest that HPB is on a saturated pathway and is produced in a rate limited step.

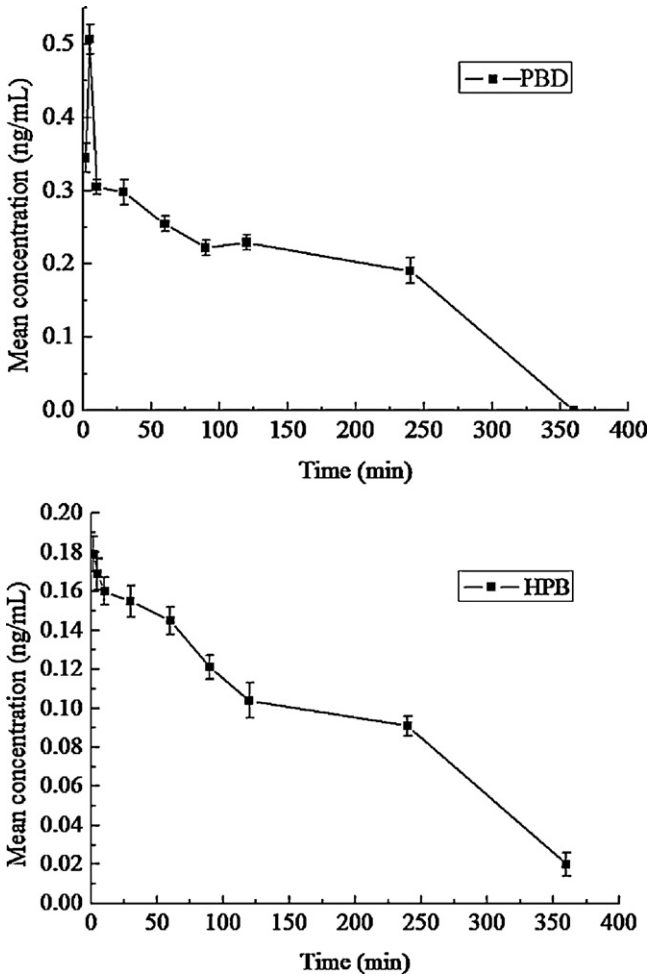

Fig. 4. (*Continued*).

As shown in Fig. 1, HPB, PBD, myosmine, and keto acid have previously been shown by other authors as products of 2′-hydroxylation of NNN, and hydroxy acid to be a metabolite of the 5′-hydroxylation pathway of NNN. There were also other two pathways including the formation of norcotinine and the Pyridine-*N*-oxidation of NNN. The metabolites' concentrations for each pathway at different time were calculated and are listed in Table 5. We observe that the total concentrations of products from the 5′-hydroxylation pathway were higher than any other pathways from 2 min to 360 min. Consequently, we conclude that under the administration conditions described, 5′-hydroxylation of NNN is the most significant pathway in the rabbit. The long plasma half-life of HPB is suggestive of a rate limitation to the 2′-hydroxylation pathway while the quantitative similarities in half-lives for norcotinine, 2′-hydroxy, and 5′-hydroxy derivative metabolites also supports that the pathways are all important but rate limited.

4. Conclusion

A simple and accurate method has been established to detect NNN and its metabolites in rabbit blood with LC–MS/MS. Metabolic curves and pharmacokinetic parameters of NNN and its metabolites were obtained showing that NNN was rapidly metabolized in rabbit blood with hydroxy acid as the major metabolite. What is more, we can infer that the 5′-hydroxylation of NNN may be the main metabolic pathway according to the datas of our study.

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