

Metabolism of Myosmine in Wistar Rats

Wolfgang Zwicknpflug, Stefan Tyroller and Elmar Richter

Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians University,
Goethestrasse 33, D-80336 Munich, Germany (W.Z., S.T., E.R.)

Short title: Metabolism of myosmine in rats

Author to whom correspondence should be addressed:

Dr. Wolfgang Zwickenpflug

Walther Straub Institute of Pharmacology and Toxicology, Ludwig-Maximilians University,

Goethestrasse 33, D-80336 Munich, Germany

E-mail: wolfgang.zwickenpflug@lrz.uni-muenchen.de

Phone: 0049-89-2180-75744

Fax: 0049-89-2180-75701

Number of text pages: 25

Number of tables: 1

Number of figures: 9

Number of references: 36

Number of words in the Abstract: 235

Number of words in the Introduction: 598

Number of words in the Discussion: 1132

Abbreviations Used

HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; hydroxy acid, 4-hydroxy-4-(3-pyridyl)butyric acid; keto acid, 4-oxo-4-(3-pyridyl)butyric acid; NNAL, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*'-nitrosonornicotine; 3-PAA, 3-pyridylacetic acid; S.D., standard deviation; SIM, selected-ion monitoring; SPE, solid phase extraction; TLC, thin layer chromatography; TSNA, tobacco-specific nitrosamines

Abstract

The alkaloid myosmine is not only present in tobacco products but also in various foods. Myosmine is easily nitrosated yielding 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) and the esophageal tobacco carcinogen *N*'-nitrosonornicotine (NNN). Due to its widespread occurrence, investigations on the metabolism and activation of myosmine are needed for risk assessment. Therefore, the metabolism of myosmine has been studied in Wistar rats treated with single oral doses of [pyridine-5-³H]myosmine at 0.001, 0.005, 0.5 and 50 $\mu\text{mol/kg}$ body weight. Oral administration was achieved by feeding a labeled apple bite. Radioactivity was completely recovered in urine and feces within 48 h. At the two lower doses, 0.001 and 0.005 $\mu\text{mol/kg}$, a higher percentage of the radioactivity was excreted in urine ($86.2 \pm 4.9\%$ and $88.9 \pm 1.7\%$) as compared to the higher doses, 0.5 and 50 $\mu\text{mol/kg}$, where only $77.8 \pm 7.3\%$ and $75.4 \pm 6.6\%$ of the dose was found in urine. Within 24 h urinary excretion of radioactivity was nearly complete with less than 4% of the total urinary output appearing between 24 and 48 h. The two major metabolites accounting for >70% of total radioactivity in urine were identified as 3-pyridylacetic acid (3-PAA, 20-26%) and 4-oxo-4-(3-pyridyl)butyric acid (keto acid, 50-63%) using UV-DAD detection and GC/MS measurements. 3-Pyridylmethanol (3-5%), 3'-hydroxymyosmine (2%) and HPB (1-3%) were detected as minor metabolites. 3'-Hydroxymyosmine is exclusively formed from myosmine and therefore might be used as urinary biomarker for myosmine exposure in the future.

For a long time, myosmine was considered as one of the minor tobacco-specific alkaloids present in mainstream smoke at 13 – 300 µg/plain nonfilter cigarette (Baker, 1999). Only little attempts were made to evaluate its possible toxicological relevance besides early investigations about nicotinoids by Werle and Schievelbein (1961). Myosmine has been detected in nuts and nut products (Zwickenpflug et al., 1998) and therefore its presence and human uptake is no longer restricted to tobacco and tobacco products. In a follow-up study, myosmine was found to be present in staple foods like wheat, rice and maize but also in fruits, vegetables and milk (Tyroller et al., 2002). Myosmine uptake from these sources was estimated to be at least as high as from exposure to environmental tobacco smoke.

In the course of the first studies on the metabolism of the tobacco-specific nitrosamines (TSNA), 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*'-nitrosonornicotine (NNN), myosmine was found as a metabolite. Myosmine has been detected after *in vitro* incubation of rat liver microsomes with NNK but was not present in rat urine after NNK administration (Hecht et al., 1980b). In contrast, myosmine was found in trace quantities as urinary metabolite of NNN in rats. To investigate the further degradation of NNN metabolites, myosmine has been administered s.c. to rats at a dose of 100 mg/kg and 4-oxo-4-(3-pyridyl)butyric acid (keto acid) was identified as main metabolite (Hecht et al., 1981).

Under nitrosation conditions myosmine forms not only NNN, an esophageal carcinogen in rats (Hecht, 1998) and recently classified as “carcinogenic to humans” by the IARC (IARC, 2004), but also to a greater extent 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) (Zwickenpflug, 2000)(Figure 1). The formation of HPB occurs presumably through an one-step reaction leading to the same reactive intermediate, an unstable diazotate, which is considered to be one of the ultimate carcinogens formed from NNN and NNK after metabolic activation by α -hydroxylation (Hecht, 1998; Hecht, 2003). In the presence of nitrite, nitrosation of myosmine in human gastric juice yielded NNN and HPB with optimal reaction

conditions at pH 2-5 (Wilp et al., 2002; Zwickenpflug, 2000). Preliminary studies on the peroxidation of myosmine yielded keto acid, 3-pyridylmethanol, HPB and normicotyrine as reaction products and therefore myosmine peroxidation could be an additional activation pathway (Tyroller and Zwickenpflug, 2005).

Myosmine was not mutagenic in the Ames assay (Riebe et al., 1982) and did not increase the spontaneous sister chromatid exchange in ovary cells after *in vivo* treatment of hamsters (Riebe and Westphal, 1983). However, in the test system *Escherichia coli* *polA*⁺/*polA*⁻ a weak mutagenic response was observed (Riebe et al., 1982). The mutagenic potential of myosmine was confirmed by the detection of DNA damage in human lymphocytes and nasal mucosa cells by the Comet assay (Kleinsasser et al., 2003). Under nitrosation conditions at pH 3-5 covalent binding of radioactivity derived from ³H-myosmine to calf thymus DNA was observed *in vitro* (Wilp et al., 2002). In a preliminary *in vivo* study HPB-releasing DNA and hemoglobin adduct formation was demonstrated by feeding myosmine to rats in combination with nitrite in the drinking water (Richter et al., 2002). In rodent experiments, HPB-releasing DNA adducts have been implicated not only in the induction of lung tumors by NNK and esophageal tumors by NNN (Hecht, 1998) but also as inhibitor of O⁶-alkylguanine-DNA alkyltransferase, the rate limiting repair enzyme of promutagenic O⁶-alkylguanine adducts (Peterson et al., 2001).

These findings suggest, that myosmine should not only be seen as minor tobacco alkaloid and as a metabolite of other tobacco derived compounds but also as a possible tobacco-independent dietary carcinogenic risk factor. Therefore, the concentration-dependent metabolism of dietary myosmine has been investigated in more detail.

Experimental Procedures

Chemicals

3-Pyridylmethanol and chemicals for synthesis of myosmine, keto acid, 3'-hydroxymyosmine and HPB were obtained from Sigma-Aldrich (Deisenhofen, Germany). [Pyridine-5-³H]myosmine, 99.9% pure, specific activity 24 Ci/mmol was purchased from Hartmann Analytic (Braunschweig, Germany). All solvents and chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). A mixture of NNN metabolite standards for HPLC was kindly donated by Dhimant H. Desai and Shantu Amin (American Health Foundation, Valhalla, NY, USA).

Synthesis of Myosmine, 3'-Hydroxymyosmine, Keto acid and HPB

Myosmine (HPLC purity >99%) and keto acid (HPLC purity >98%) were synthesized according to Brandänge and Lindblom (1976) and McKennis et al. (1964b), respectively. Synthesis of HPB (HPLC purity >99%) and 3'-hydroxymyosmine (HPLC purity >97%) was performed in accordance to Hecht et al. (1977) and Hecht et al. (1980a), respectively. The physicochemical parameters of the products were in accordance with values reported in the literature.

Animal Experiment

Female Wistar rats (200-250 g) from Harlan-Winkelmann (Borchen, Germany) were housed in stainless steel cages with a 12-h light/dark cycle. The temperature was maintained at 22 ± 1 °C; the relative humidity was between 50 and 60% and fresh air was exchanged 100% from 18 to 22 times every hour. Food (ssniff R, ssniff, Soest, Germany) and water was provided *ad libitum*. For collection of urine and feces the rats were housed individually in stainless steel metabolism cages. After 24 h adaptation, exposure to myosmine was managed by feeding groups of 4 rats/dose contaminated apple bites. Each bite was prepared with 2

pmol [pyridine-5-³H]myosmine and unlabeled myosmine to achieve doses of 0.001, 0.005, 0.5 and 50 µmol/kg body weight. One rat of the highest dose did not take the bait. Urine was collected at 6, 24 and 48 h after exposure. Feces were pooled to give one 48 h sample. For identification of metabolites four rats were fed 2 mg/rat (68.4 µmol/kg body weight) of unlabeled myosmine each and urine was collected for 24 h. The animal experiments were officially approved by the Government of Upper Bavaria (AZ 211-2531-79/99).

Measurement of Total Radioactivity in Urine and Feces

Triplicate 50 µl urine samples were mixed with 10 ml of Ultima Gold XR scintillator (Packard, Frankfurt/Main, Germany) for determination of total radioactivity in a Tri Carb 2500 TR Liquid Scintillation Analyzer (Packard, Frankfurt/Main, Germany). Feces were mixed with 2.5 ml methanol/g wet weight and homogenized with an Ultra-Turrax (Bachofer, Reutlingen, Germany). To 0.1 g of the homogenates 0.2 ml hydrogen peroxide and 0.2 ml perchloric acid were added and incubated at 80 °C for 30 min. After cooling, 10 ml liquid scintillator was added to the samples which were measured after overnight storage in the dark.

Clean up of Urine Samples for Metabolite Identification

After centrifugation aliquots of 1-2 ml urine from rats fed with 2 mg unlabeled myosmine were adjusted to pH 7 and transferred to a preconditioned 3 ml C₁₈ solid-phase extraction (SPE) tube with stainless steel frits and 500 mg of sorbent material (Varian GmbH, Darmstadt, Germany). Retained myosmine metabolites were successively eluted with 2 ml CH₃CN/H₂O (2:98, v/v), 2 ml CH₃CN/H₂O (4:96, v/v), 2 ml CH₃CN/H₂O (6:94, v/v) and 2 ml CH₃CN/H₂O (8:92, v/v).

Derivatisation of 3-Pyridylacetic acid Standard, Keto acid Standard and of an Urine Fraction

To 0.01 mmol (1.37 mg) 3-pyridylacetic acid dissolved in 5 ml acetone, 0.03 mmol (4.15 mg) potassium carbonate was added. After stirring the reaction mixture at room temperature for 10 min, 0.03 mmol (4.26 mg) CH₃I was added. The reaction mixture was stirred under reflux for another 3 h. After removal of the acetone at reduced pressure the residue was taken up in CH₂Cl₂ and analyzed by GC/MS yielding corresponding signals as reported by Pilotti et al. (1976).

4-Oxo-4-(3-pyridyl)butyric acid (0.015 mmol, 2.69 mg) was dissolved in 5 ml acetone. Potassium carbonate (0.04 mmol, 5.53 mg) was added and the reaction mixture was stirred for 10 min. CH₃I (0.04 mmol, 5.68 mg) was added and the reaction mixture was stirred under reflux for 4 h. The solvent was removed at reduced pressure. The residue was dissolved in CH₂Cl₂ and analyzed by GC/MS. The obtained spectra were equal to those in the literature (Pilotti et al., 1976).

Both, the urine passing through the tube (containing 3-pyridylacetic acid) and the fraction eluted with 2 ml CH₃CN/H₂O (2:98, v/v) containing keto acid, were concentrated under reduced pressure and derivatised as described above. The reaction mixture containing methylated 3-pyridylacetic acid was further cleaned up by thin layer chromatography (TLC) on an analytical Kieselgel F254 plate (Merck, Darmstadt, Germany) using CHCl₃/MeOH (10:1, v/v). The spot with R_f 0.7 was removed from the plate and eluted with CHCl₃/MeOH (3:2, v/v).

Derivatisation of 3-Pyridylmethanol Standard and of an Urine Fraction

To 3-pyridylmethanol standard 1 ml NMe₃/CH₂Cl₂ (1:1, v/v) and 10 µl of a pentafluorobenzoyl chloride solution (10 µl PFBC and 490 µl NMe₃/CH₂Cl₂ (1:1, v/v)) were added. After stirring, the reaction mixture was heated at 60 °C for 2 h. The solvent was

removed in a vacuum concentrator. Hexane (80 μ l) was added and centrifuged at 2000 g for 10 min.

An aliquot of a urine fraction ($\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (4:96, v/v)) was cleaned up by 2-dimensional thin layer chromatography (TLC) on an analytical Kieselgel F254 plate (Merck, Darmstadt, Germany) using $\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$ (85:15:2, v/v) followed by $\text{CHCl}_3/\text{MeOH}$ (3:2, v/v). The region with corresponding R_f values to 3-pyridylmethanol standard was removed from the plate and eluted with $\text{CHCl}_3/\text{MeOH}$ (3:2, v/v). After removal of the solvent the residue was further derivatized as aforementioned.

Isolation of 3'-Hydroxymyosmine

3'-Hydroxymyosmine was located in the urine fraction $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (8:92, v/v). An aliquot of the fraction was transferred on an analytical Kieselgel F254 plate ($\text{CHCl}_3/\text{MeOH}/\text{NH}_4\text{OH}$; 85:15:2, v/v). The spot with R_f 0.46 was removed from the plate and extracted with $\text{CHCl}_3/\text{MeOH}$ (3:2, v/v). The residue was further analyzed by LC/MS.

HPLC Measurements

Urine samples of 400 μ l were filtered by centrifugation at 2200 g through a 0.2 μ m Nanosep MF filter (Pall, Dreieich, Germany) and analyzed by HPLC with online radioactivity monitoring and scintillator pump (Ramona 2000, Raytest, Straubenhardt, Germany) adding 3 ml/min Quickszint Flow 302 liquid scintillator (Zinsser Analytic, Frankfurt, Germany). Separations were carried out on a Gynkotek HPLC system (Dionex, Idstein, Germany) using a 250 \times 4 mm Lichrospher 60 RP-18 SelectB 5 μ column and a 4 \times 4 mm LiChroCart guard column filled with LiChrospher 100 RP-18 5 μ (Merck, Darmstadt, Germany). The column was operated at a flow rate of 0.7 ml/min with a gradient using acetonitrile and 15 mM ammonium acetate buffer. After an initial time of 5 min at 1% $\text{CH}_3\text{CN}/99\%$ buffer, CH_3CN was linearly increased over 15 min up to 18% and held for another 15 min. Within 5 min the

eluent was changed to 60% CH₃CN/40% buffer, held for 2 min, and returned to 1% CH₃CN/99% buffer within 2 min. The column was reconditioned for at least 10 min. At the flow rate of 0.7 ml/min a time delay of about 0.6 min occurred between detection of the UV and radioactivity signal. The chromatograms were recorded at wavelengths of 232 and 254 nm. For identification of the metabolites UV spectra were recorded by a UVD 320A diode array detector (Dionex, Idstein, Germany). The operating wavelength of the diode array system for spectra recording was adjusted to 270 nm with a bandwidth of 140 nm (200-340 nm).

GC/MS and LC/MS Measurements

Analytical assessment of the derivatized metabolites was performed using a Fisons 8060 gas chromatograph coupled to a TRIO 1000 quadrupole mass selective detector (Fisons Instruments, Mainz, Germany) in the electron impact mode at 70 eV. Separation was executed on a ZB50 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Phenomenex (Aschaffenburg, Germany) with helium as carrier gas. The oven temperature was held at 80 °C for 1 min, then increased to 310 °C at 15 °C/min and held for 5 min. The temperatures of the injection port, transfer line, and ion source were set at 200, 300 and 250 °C. All ions were monitored with a dwell time of 100 ms. In the scan mode, masses in the range of 50 and 200 amu were acquired. For detection of 3-pyridylacetic acid methyl ester and derivatized 3-pyridylmethanol in the SIM mode the molecular ions *m/z* 65, 92, 107, 151 and 65, 92, 108, 117, 167, 195, 303 were monitored, respectively.

LC/MS measurements of 3'-hydroxymyosmine were carried out on an Agilent 1100 Series HPLC (Böblingen, Germany) coupled with a Sciex API 2000 (Applied Biosystems, Darmstadt, Germany). The HPLC was operated without a column at a flow rate of 0.2 ml/min using methanol/acetonitrile/0.1% formic acid (50:48:2, v/v). The spray voltage was 5.5 kV and the capillary temperature 350 °C by an injection volume of 5 μl.

Statistical analysis

Reported values represent mean \pm standard deviation (S.D.). Differences between the groups were tested for statistical significance by the two sided *t*-test for independent samples using WINSTAT for MICROSOFT EXCEL (R. Fitch Software, Staufen, Germany).

Results

Using a contaminated apple bite is a practical way of myosmine administration to ensure that a defined amount of myosmine is picked up. The animals are not stressed by injection or gavage and receive the test material in the most natural way taking into account the widespread occurrence of myosmine in food. Extraction of a contaminated apple bite with $\text{CHCl}_3/\text{MeOH}$ (3:2, v/v) resulted in total recovery of labeled myosmine without any degradation.

Elimination of Myosmine

Between 87.9 ± 21.1 and 100.5 ± 26.8 percent of the radioactive dose was recovered in urine and feces (Table I). The relative amounts of total radioactivity excreted in urine and feces were dose-dependent. At the two lower doses, a higher percentage of radioactivity, $86.2 \pm 4.9\%$ and $88.9 \pm 1.7\%$, was recovered in urine as compared to the two higher doses where only $77.8 \pm 7.3\%$ and $75.4 \pm 6.6\%$ appeared in the urine.

Within the first 6 h after myosmine administration $88.5 \pm 2.5\%$ (0.001 $\mu\text{mol/kg}$), $87.3 \pm 7.6\%$ (0.005 $\mu\text{mol/kg}$), $79.0 \pm 22.1\%$ (0.5 $\mu\text{mol/kg}$) and $78.1 \pm 7.9\%$ (50 $\mu\text{mol/kg}$) of the total radioactivity excreted in urine was recovered. After 24 h $97.4 \pm 1.4\%$ (0.001 $\mu\text{mol/kg}$), $98.1 \pm 0.4\%$ (0.005 $\mu\text{mol/kg}$), $97.3 \pm 1.1\%$ (0.5 $\mu\text{mol/kg}$) and $95.2 \pm 6.3\%$ (50 $\mu\text{mol/kg}$) were detected in the urine. Thus urinary excretion is nearly complete within the first 24 h.

Metabolite Spectrum

A typical chromatogram of 6-h urinary metabolites is shown in Figure 2. Besides two major peaks three minor metabolites of myosmine were observed corresponding to 84-91% of total radioactivity recovered from HPLC. No unmetabolised myosmine was detected in the urine at its retention time of 46 min. The retention times of the minor metabolites did not match any of the known major metabolites of NNN such as hydroxy acid, diol, norcotinine,

normicotine or NNN-*N*-oxide. The two major myosmine metabolites could be identified (see below) as 3-PAA and keto acid (Figure 1). 3-PAA contributed to a similar percentage to the sum of all urinary metabolites averaging $23.2 \pm 2.6\%$ over the whole concentration range (Table I). In contrast, the percentage of keto acid increased significantly at the highest dose ($63.1 \pm 2.9\%$) compared to the three lower doses ($52.0 \pm 2.3\%$ on average, $P < 0.05$). The minor metabolites 3-pyridylmethanol (3.1-5.3%), 3'-hydroxymyosmine (1.6-2.4%) and HPB (1.6-3.1%) accounted for less than 10% of total radioactivity in urine (Table I, Figure 1).

Identification of the Metabolites

For identification of pH-sensitive groups at the hydrophilic metabolites, the pH of the HPLC buffer was decreased to look for a possible shift in retention times. After adjusting the buffer to pH 4, only the main metabolite moved to a later retention time. This indicates the presence of at least one carboxyl group in the molecule. Reducing the pH further to search for stronger acids the chromatography became irreproducible. The main metabolite was identified as the keto acid by co-elution with a standard (see Figure 3 for correlation of DAD-UV- and radio-HPLC retention times). Comparison of the UV spectra confirmed the assignment (Figure 4). Last evidence was obtained by GC/MS measurements after converting the keto acid to its methyl ester (Figure 5).

Attempts to identify the second largest signal headed towards stronger acids than the keto acid. Nicotinic acid as well as 3-PAA showed no shift in the retention time at pH 4. Whereas the UV spectra of the two standards and of the corresponding signal were almost identical, only 3-PAA co-eluted with the corresponding peak in HPLC (see Figure 4 for DAD-UV spectra). Further evidence was obtained by GC/MS analysis of the methylated compound. Prior to the methylation reaction, the urine sample had to be cleaned from major impurities by C₁₈ solid-phase extraction. Radioactivity associated with hydrophilic 3-PAA was almost completely recovered in the break through fluid and most of the substances

interfering in the later GC/MS analysis were retained at the C₁₈ material. After methylation and further clean-up by TLC, GC-chromatograms and MS-spectra demonstrate the presence of 3-pyridylacetic acid methyl ester (Figure 6).

The urine fraction received after elution of the SPE tube with CH₃CN/H₂O 4:96 contained 3-pyridylmethanol. Identification is based on coelution with a standard on HPLC (Figure 7), UV spectra (Figure 4) and GC/MS data after derivatization with pentafluorobenzoyl chloride (Figure 8). 3'-Hydroxymyosmine was present in the urine fraction eluted with CH₃CN/H₂O 8:92 and was identified by HPLC-UV (Figures 4 and 7) and LC/MS (Figure 9). The identity of HPB was verified by co-elution with an unlabeled standard and comparison of the UV-spectra (Figure 4; For correlation of HPLC-UV and radioHPLC retention times see Figure 3).

Discussion

Myosmine was administered over a very broad range of doses with the lowest dose approaching the estimated human myosmine uptake from food or passive smoke exposure (Tyroller et al., 2002). Oral administration was chosen to simulate real conditions of dietary myosmine uptake allowing contact with oral cavity and esophageal tract. Myosmine given to rats over a dose range of 0.001-50 $\mu\text{mol/kg}$ body weight undergoes rapid and complete metabolism. The absence of unmetabolised myosmine in urine confirms earlier studies in humans taking orally 2 mg of myosmine (Beckett et al., 1971) and is in contrast to results for nicotine and nornicotine in rats and humans (Schepers et al., 1993; Hukkanen et al., 2005; Beckett et al., 1971). Apart from 3'-hydroxymyosmine, the identified metabolites in rat urine (Figure 1) are in part well known from metabolism studies with other tobacco nicotinoids and TSNA. HPB and keto acid are metabolites of NNK, NNN and nicotine. 3-PAA was identified as urinary metabolite in animals or man after administration of nicotine, cotinine and keto acid (McKennis et al., 1964a; McKennis et al., 1964b; Schepers et al., 1993). First reports on the occurrence of nicotine derived 3-pyridylmethanol in smokers urine were speculative (Barlow et al., 1987; Parviainen and Puhakainen, 1990). Further evidence was given by Rop et al. (1993) using HPLC-UV-DAD. However, the presence of all four metabolites together and the high amounts of 3-PAA relative to keto acid are unique for myosmine. These findings might be ascribed to a different way of administration, dosage, metabolism in different organs or tissues, but mainly to the physico-chemical properties of the compound itself. Myosmine is the only known tobacco alkaloid with an imine structure. This enables myosmine to undergo a pH dependent ring-chain tautomerism which can offer new metabolic pathways and even direct chemical reactions. Under acidic conditions at pH <6 the open amino ketone form is already dominating and at pH <3 less than 10% is detectable in the ring form (Brandänge and Rodriguez, 1983).

The high percentage of 3-PAA and the observed ratio of 3-PAA to keto acid in myosmine metabolism was unexpected. In phase 1 metabolism of nicotine 3-PAA is one of the end products. 4-Hydroxy-4-(3-pyridyl)butyric acid (hydroxy acid) and 4-(3-pyridyl)butyric acid besides 3-PAA have been isolated and identified as metabolites of keto acid partially in rats, rabbits and dogs (McKennis et al., 1964b). Although the metabolic pathway leading from keto acid to 3-PAA has not been cleared up in detail, it is commonly assumed that 3-PAA is formed from hydroxy acid by dehydration to 4-(3-pyridyl)-3-butenic acid. Reduction of the butenoic double bond leads to 4-(3-pyridyl)-butyric acid and following β -oxidation (via Knoop-degradation) yields 3-PAA (McKennis et al., 1964a; Meacham et al., 1972). Even 40 years after these first assumptions 3-PAA formation remains still speculative (Hukkanen et al., 2005). Studies on the metabolism of (E)-metanicoline pointed out 4-(3-pyridyl)-3-butenic acid as a major and 3-PAA as a terminal metabolite (Bencherif et al., 1997). The postulated role of keto acid and hydroxy acid as precursors of 3-PAA is weakened by the fact that 3-PAA has not been identified as metabolite of NNN and NNK although keto acid and hydroxy acid are the major urinary metabolites of these two TSNA in all species investigated so far (Hecht, 1998).

Keto acid, accounting for >50% of the radioactivity in rat urine, has been earlier described as myosmine metabolite in rats (Hecht et al., 1981). It is also a major urinary metabolite of NNN and NNK in rodents and primates where it arises from further oxidation of primary α -hydroxylation products of both TSNA (Hecht, 1998). Considering that *in vitro* nitrosation of myosmine under simulated gastric conditions yields up to 72% and 16% of HPB and NNN (Wilp et al., 2002), respectively, HPB and keto acid in urine of myosmine-treated rats could arise from *in vivo* nitrosation of myosmine either directly or subsequent to metabolic activation of NNN (McIntee and Hecht, 2000; Hecht et al., 1981). In contrast to myosmine, small amounts of NNN are excreted unchanged in rat urine. However, neither NNN nor hydroxy acid, a major metabolite of NNN accounting for 44% of the dose in rats

(Tyroller et al., 2004), have been identified in rat urine after myosmine administration. Therefore, no evidence for *in vivo* formation of NNN from myosmine was found in the present study. In this context, only 1.1% of [5-³H]keto acid i.v. administered to F344 rats was metabolized to hydroxy acid whereas 94.8% of the keto acid was excreted unmetabolised (Trushin and Hecht, 1999). In case that hydroxy acid is exclusively formed from keto acid, the percentage expected in myosmine metabolism would be approximately 0.5% of all metabolites and therefore below the detection limit in the present study.

Contribution of myosmine nitrosation to HPB formation in rats must be discussed differentiated. Although no NNN was found in rat urine and therefore *in vivo* nitrosation seems to contribute only little to myosmine metabolism, HPB and its postulated oxidation product keto acid were detected in rat urine accounting for 52-66% of total radioactivity recovered from HPLC. Another activation pathway except nitrosation might be responsible for *in vivo* HPB formation. In preliminary studies on the *in vitro* peroxidation of myosmine, keto acid, 3-pyridylmethanol and HPB were identified as reaction products (Tyroller and Zwicklenpflug, 2005). Interestingly, all three compounds are also present in urine of myosmine treated rats. Therefore, it can be assumed that biochemical activation of myosmine e.g. by P450 oxidation is not the only pathway and direct chemical activation might contribute to a significant extent. Myosmine offers different sites for P450 oxidation reactions. 5'-Hydroxylation might lead to an instable intermediate whose fate has not been cleared up so far. A major enzyme metabolizing nicotine, human CYP 2A6, was only weakly inhibited by myosmine and therefore may not play a major role in myosmine metabolism (Denton et al., 2004). Oxidation (not necessarily P450 mediated) of the imine double bond might result in the formation of keto acid, 3-pyridylmethanol and HPB (identified after *in vitro* peroxidation). No 4'-hydroxymyosmine was present in rat urine and only small amounts of 3'-hydroxymyosmine were found. But 3'-hydroxymyosmine is formed from myosmine exclusively and therefore it could be used as an urinary biomarker for myosmine exposure.

The formation of HPB-releasing DNA adducts which were found in tissues from myosmine treated rats (Richter et al., 2002) may not be the only activation mechanism of myosmine. 3-PAA formation after oral administration of myosmine to rats may also include reactive intermediates leading to different protein and DNA adducts. To elucidate a possible alkylation of biomolecules caused by myosmine metabolism implying a toxicological relevance, further studies are necessary. Especially endogenous nitrosation as well as peroxidative activation of the closed imine-structure as indicated in our previous studies, but also oxidative desamination of the aminoketone form or enzymatic activation should be taken into consideration.

References

- Baker RR, 1999. Smoke chemistry. In: Davis, D.L., Nielsen, M.T. (Eds.) Tobacco production, chemistry and technology. Blackwell Science Ltd., Oxford, pp. 398-439.
- Barlow RD Thompson PA and Stone RB (1987) Simultaneous determination of nicotine, cotinine and five additional nicotine metabolites in the urine of smokers using pre-column derivatisation and high-performance liquid chromatography. *J Chromatogr* **419**:375-380.
- Beckett AH Gorrod JW and Jenner P (1971) A possible relation between pKa1 and lipid solubility and the amounts excreted in urine of some tobacco alkaloids given to man. *J Pharm Pharmacol* **24**:115-120.
- Bencherif M Byrd G Caldwell WS Hayes JR and Lippiello PM (1997) Pharmacological Characterization of RJR-2403: A nicotinic agonist with potential therapeutic benefit in the treatment of Alzheimer's disease. *CNS Drug Reviews* **3**:325-345.
- Brandänge S and Lindblom L (1976) *N*-Vinyl as N-H protection group. A convenient synthesis of myosmine. *Acta Chem Scand* **30**:39.
- Brandänge S and Rodriguez B (1983) Ring chain tautomerism of myosmine. *Acta Chem Scand Ser B* **37**:643-644.
- Denton TT Zhang X and Cashman JR (2004) Nicotine-related alkaloids and metabolites as inhibitors of human cytochrome P-450 2A6. *Biochem Pharmacol* **67**:751-756.
- Hecht SS (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. *Chem Res Toxicol* **11**:559-603.
- Hecht SS (2003) Tobacco carcinogens, their biomarkers and tobacco-induced cancer. *Nature Rev Cancer* **3**:733-744.
- Hecht SS Chen CB Dong M Orna RM Hoffmann D and Tso TC (1977) Chemical studies on tobacco smoke LI: studies on non-volatile nitrosamines in tobacco. *Beitr Tabakforsch* **9**:1-6.
- Hecht SS Chen C-H and Hoffmann D (1980a) Metabolic β -hydroxylation and N-oxidation of *N*'-nitrosornicotine. *J Med Chem* **23**:1175-1178.
- Hecht SS Lin D and Chen CB (1981) Comprehensive analysis of urinary metabolites of *N*'-nitrosornicotine. *Carcinogenesis* **2**:833-838.
- Hecht SS Young R and Chen CB (1980b) Metabolism in the F344 rat of 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. *Cancer Res* **40**:4144-4150.
- Hukkanen J Jacob P and Benowitz NL (2005) Metabolism and disposition kinetics of nicotine. *Pharmacol Rev* **57**:79-115.
- IARC. IARC Monographs Programme Finds Smokeless Tobacco Is Carcinogenic To Humans. Press Release No 154. 2004. Lyon, International Agency for Research on Cancer.

- Kleinsasser NH Wallner BC Harréus UA Zwickenpflug W and Richter E (2003) Genotoxic effects of myosmine in human lymphocytes and upper aerodigestive tract epithelial cells. *Toxicology* **192**:171-177.
- McIntee EJ and Hecht SS (2000) Metabolism of *N*'-nitrosonornicotine enantiomers by cultured rat esophagus and in vivo in rats. *Chem Res Toxicol* **13**:192-199.
- McKennis H Schwartz SL and Bowman ER (1964a) Alternate routes in the metabolic degradation of the pyrrolidine ring of nicotine. *J Biol Chem* **239**:3990-3996.
- McKennis H Schwartz SL Turnbull LB Tamaki E and Bowman ER (1964b) The metabolic formation of γ -(3-pyridyl)- γ -hydroxybutyric acid and Its possible intermediary role in the mammalian metabolism of nicotine. *J Biol Chem* **239**:3981-3989.
- Meacham RH Bowman ER and McKennis H (1972) Additional routes in the metabolism of nicotine to 3-pyridylacetate. *J Biol Chem* **247**:902-908.
- Parviainen MT and Puhakainen EVJ (1990) Nicotine metabolites in the urine of smokers. *J Chromatogr* **525**:193-202.
- Peterson LA Thomson NM Crankshaw DL Donaldson EE and Kenney PJ (2001) Interactions between methylating and pyridyloxobutylating agents in A/J mouse lungs: Implications for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis. *Cancer Res* **61**:5757-5763.
- Pilotti A Enzell CR McKennis H Bowman E Dufva E and Holmstedt B (1976) Studies on the identification of tobacco alkaloids, their mammalian metabolites and related compounds by gas chromatography-mass spectrometry. *Beitr Tabakforsch* **8**:339-349.
- Richter E Schlöbe D Hölzle D and Wilp J (2002) Comparative DNA and hemoglobin adduct formation by dietary myosmine and *N*'-nitrosonornicotine in rats. *Naunyn Schmiedebergs Arch Pharmacol* **365** (Suppl.1):R140.
- Riebe M and Westphal K (1983) Studies on the induction of sister-chromatid exchanges in Chinese hamster ovary cells by various alkaloids. *Mutat Res* **124**:281-286.
- Riebe M Westphal K and Fortnagel P (1982) Mutagenicity testing, in bacterial test systems, of some constituents of tobacco. *Mutat Res* **101**:39-43.
- Rop PP Grimaldi F Oddoze C and Viala A (1993) Determination of nicotine and its main metabolites in urine by high-performance liquid chromatography. *J Chromatogr B* **612**:302-309.
- Schepers G Rustemeier K Walk R-A and Hackenberg U (1993) Metabolism of S-nicotine in noninduced and Aroclor-induced rats. *Eur J Drug Metab Pharmacokinet* **18**:187-197.
- Trushin N and Hecht SS (1999) Stereoselective metabolism of nicotine and tobacco-specific *N*-nitrosamines to 4-hydroxy-4-(3-pyridyl)butanoic acid in rats. *Chem Res Toxicol* **12**:164-171.
- Tyroller S and Zwickenpflug W (2005) Peroxidation of myosmine: Identification of reaction products and influence of the reaction conditions. *Proc Am Assoc Cancer Res* **46**:500.

Tyroller S Zwickenpflug W and Richter E (2002) New sources of dietary myosmine uptake from cereals, fruits, vegetables and milk. *J Agric Food Chem* **50**:4909-4915.

Tyroller S Zwickenpflug W and Richter E (2004) Interactions of different tobacco-alkaloids and TSNA on NNN-metabolism in F344 rats. *Proc Am Assoc Cancer Res* **45**:741.

Werle E and Schievelbein H (1961) Tabellen zur Toxikologie, Pharmakologie und Chemie von Inhaltsstoffen des Tabaks und des Tabakrauchs. 1. Mitteilung. *Arzneimittelforsch* **11**:1011-1016.

Wilp J Zwickenpflug W and Richter E (2002) Nitrosation of dietary myosmine as risk factor of human cancer. *Food Chem Toxicol* **40**:1223-1228.

Zwickenpflug W (2000) *N*-nitrosation of myosmine yields HPB (4-hydroxy-1-(3-pyridyl)-1-butanone) and NNN (*N*-nitrosornicotine). *J Agric Food Chem* **48**:392-394.

Zwickenpflug W Meger M and Richter E (1998) Occurrence of the tobacco alkaloid myosmine in nuts and nut products of *Arachis hypogaea* and *Corylus avellana*. *J Agric Food Chem* **46**:2703-2706.

Footnotes

This work was supported by the Deutsche Forschungsgemeinschaft (Project No. ZW 59/2-1).

Legends for Figures

Figure 1: *In vitro* nitrosation of myosmine and identified metabolites in rat urine after treatment with [pyridine-5-³H]myosmine.

Figure 2: HPLC radiochromatogram of urine from a Wistar rat treated with [pyridine-5-³H]myosmine.

Figure 3: HPLC UV-chromatogram (A) recorded at a wavelength of 232 nm and HPLC radiochromatogram (B) showing the correlation of the retention times of 3-pyridylacetic acid, keto acid and HPB. A time delay of approximately 0.6 min for the radioactive signal has to be taken into account for the analytical setting with the UV detector upstream of the radioactivity monitor.

Figure 4: UV spectra of standards (1) and from urine sample extracts (2):
(A) keto acid; (B) 3-pyridylacetic acid; (C) 3-pyridylmethanol; (D) 3'-hydroxymyosmine; (E) HPB

Figure 5: GC/MS chromatogram of keto acid methyl ester standard (A) and the related mass spectrum (B) recorded in the scan mode showing the relevant ions m/z 51, 78, 106, 134, 162 and 193. GC/MS chromatogram of keto acid methyl ester from a methylated urine sample (C). The mass spectrum (D) was recorded in the scan mode showing all relevant ions.

Figure 6: GC/MS chromatogram of 3-pyridylacetic acid methyl ester standard (A) and the related mass spectrum (B) recorded in the scan mode showing the relevant ions m/z 65, 92, 107 and 151. GC/MS chromatogram of 3-pyridylacetic acid methyl ester from a methylated urine sample (C). The mass spectrum (D) was recorded in the SIM mode showing all relevant ions.

Figure 7: HPLC UV-chromatograms of 3-pyridylmethanol- and 3'-hydroxymyosmine standard (A). HPLC UV-chromatogram of an urine fraction representing 3-

pyridylmethanol (B) and HPLC UV-chromatogram of an urine fraction representing 3'-hydroxymyosmine (C)

Figure 8: GC/MS chromatogram (m/z 303) of 3-pyridylmethanol standard after derivatization with pentafluorobenzoyl chloride (A) and the related mass spectrum (B) recorded in the SIM mode showing the relevant ions m/z 65, 92, 108, 117, 167, 195 and 303. GC/MS chromatogram (m/z 303) of derivatized 3-pyridylmethanol from an urine sample (C). The mass spectrum (D) was recorded in the SIM mode showing all relevant ions.

Figure 9: LC/MS spectra of 3'-hydroxymyosmine standard (A) and from an urine sample (B).

Tables

Table I: Time- and dose-dependent elimination of [pyridine-5-³H]myosmine in urine and feces of Wistar rats^a

| Dose ($\mu\text{mol/kg}$ bw) | Urine ^b | | | | Feces ^b | Total ^c | Urinary metabolites ^d | | | | |
|-------------------------------------|-----------------------------|----------------------------|---------------|-----------------------------|-----------------------------|--------------------|----------------------------------|-----------------------------|----------------------------|-----------------------------|----------------------------|
| | 0-6 h | 6-24 h | 24-48 h | 0-48 h | 0-48 h | 0-48 h | 3-Pyridyl- acetic acid | Keto acid | 3-Pyridyl- methanol | 3'-Hydroxy- myosmine | HPB |
| 0.001 | 76.9 \pm 4.4 ^e | 7.8 \pm 1.7 ^e | 1.5 \pm 0.8 | 86.2 \pm 4.9 | 13.8 \pm 4.9 | 100.5 \pm 26.8 | 22.0 \pm 2.0 ^f | 54.6 \pm 4.0 ^e | 4.3 \pm 0.7 ^e | 2.4 \pm 0.4 ^{ef} | 2.3 \pm 0.1 ^f |
| 0.005 | 77.9 \pm 6.4 ^e | 9.7 \pm 6.5 | 1.3 \pm 0.5 | 88.9 \pm 1.7 ^e | 11.1 \pm 1.7 ^e | 100.2 \pm 16.4 | 26.1 \pm 2.0 ^e | 51.5 \pm 3.9 ^e | 4.1 \pm 1.0 | 1.7 \pm 0.5 | 1.6 \pm 0.5 ^g |
| 0.5 | 61.5 \pm 17.8 | 14.5 \pm 17.3 | 1.8 \pm 0.8 | 77.8 \pm 7.3 | 22.2 \pm 7.3 | 87.9 \pm 21.1 | 24.6 \pm 0.6 | 50.0 \pm 2.1 ^e | 5.3 \pm 0.8 ^g | 2.0 \pm 0.3 ^e | 1.9 \pm 0.9 |
| 50 | 59.8 \pm 11.0 | 12.9 \pm 1.4 | 2.7 \pm 3.3 | 75.4 \pm 6.6 | 24.6 \pm 6.6 | 96.7 \pm 26.7 | 20.2 \pm 2.6 | 63.1 \pm 2.9 | 3.1 \pm 0.3 | 1.6 \pm 0.2 | 3.1 \pm 0.4 |

^a Mean \pm S.D. of 4 rats (at 50 $\mu\text{mol/kg}$: n = 3)

^b Percent of total recovered radioactivity in urine and feces

^c Percent of total administered radioactivity

^d Percent of total recovered radioactivity in HPLC

^e Statistically significantly different from animals fed with 50 $\mu\text{mol/kg}$, $P < 0.05$

^f Statistically significantly different from animals fed with 0.005 $\mu\text{mol/kg}$, $P < 0.05$

^g Statistically significantly different from animals fed with 50 $\mu\text{mol/kg}$, $P < 0.01$

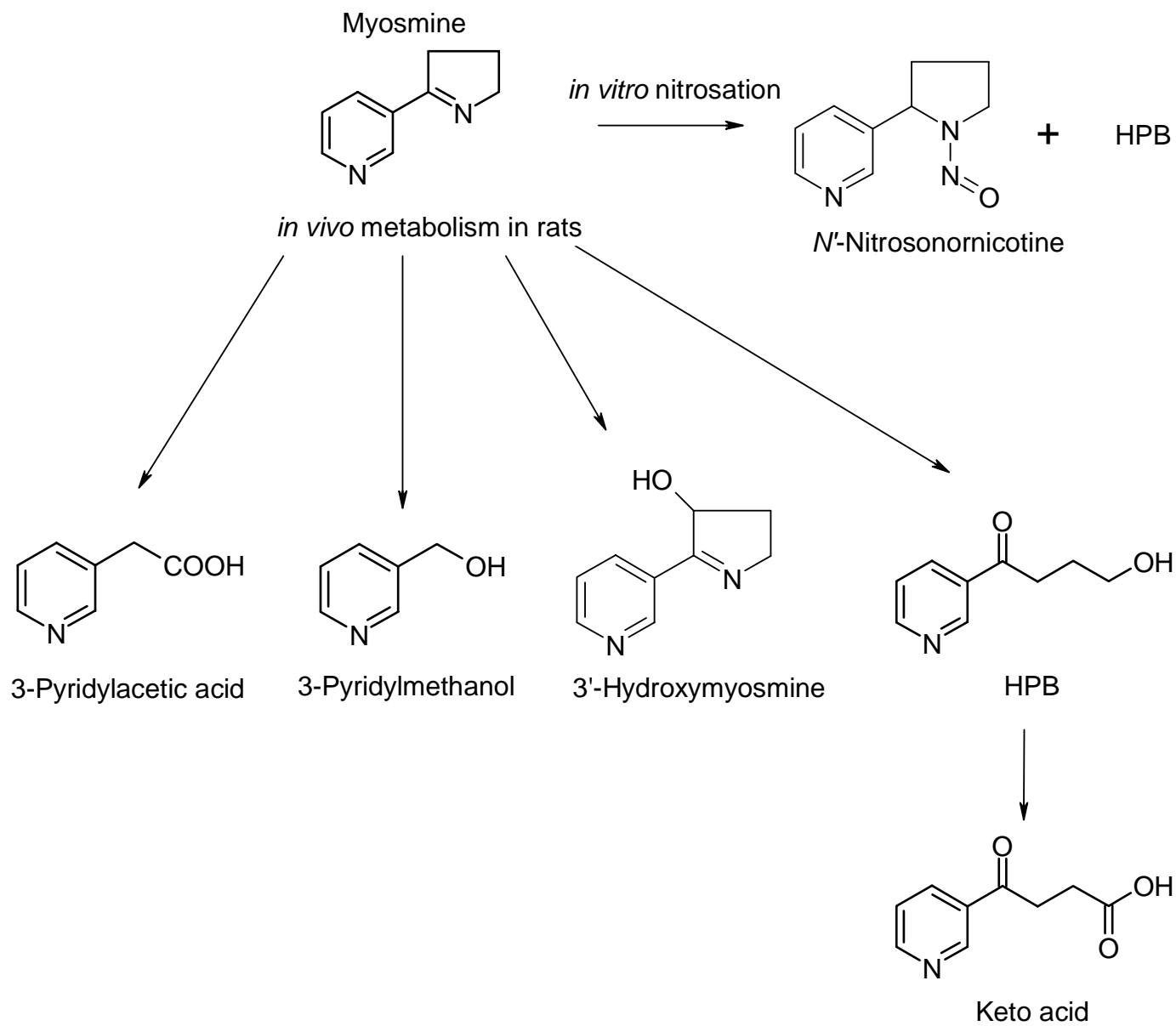


Figure 1:

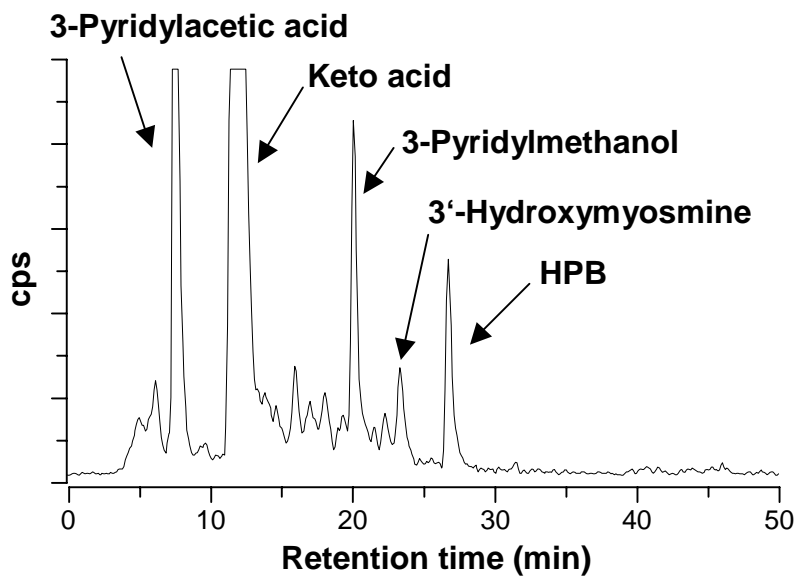


Figure 2:

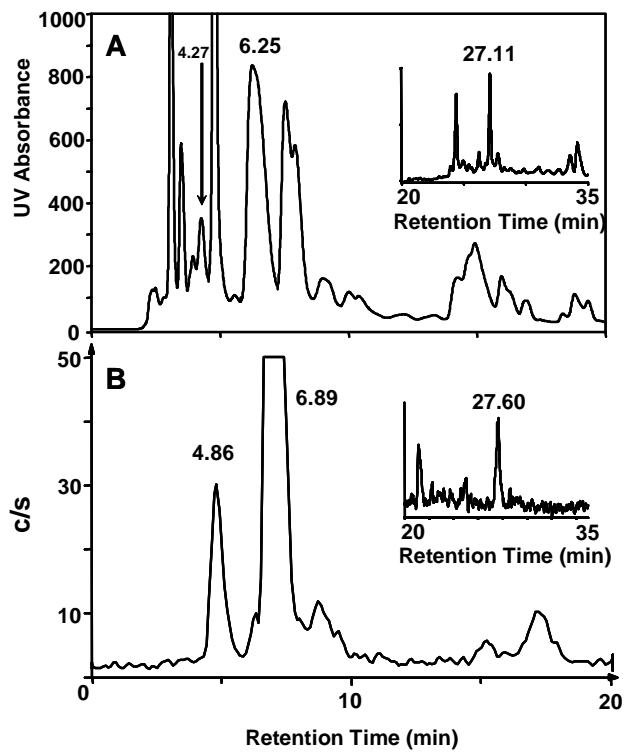


Figure 3:

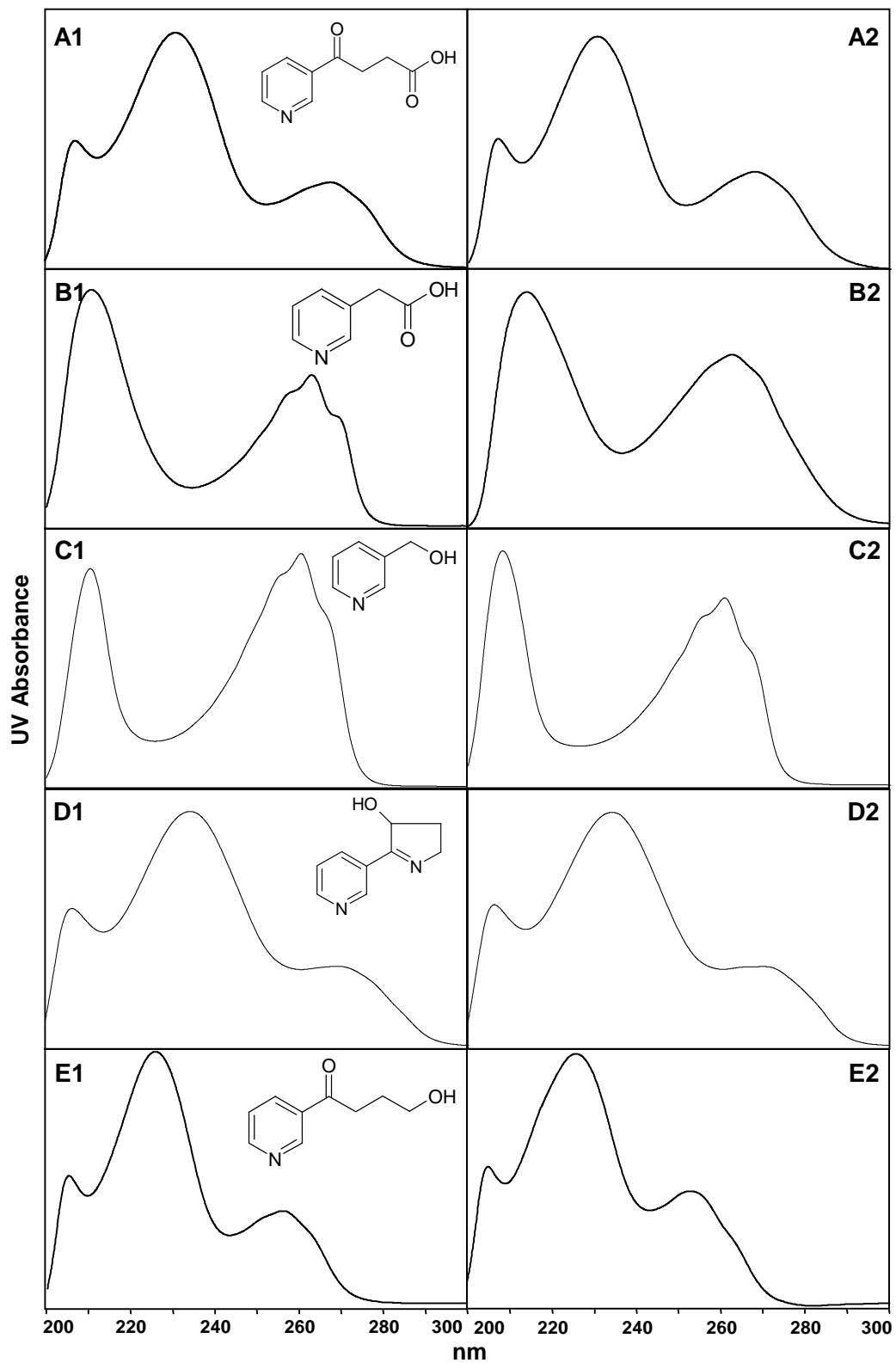


Figure 4:

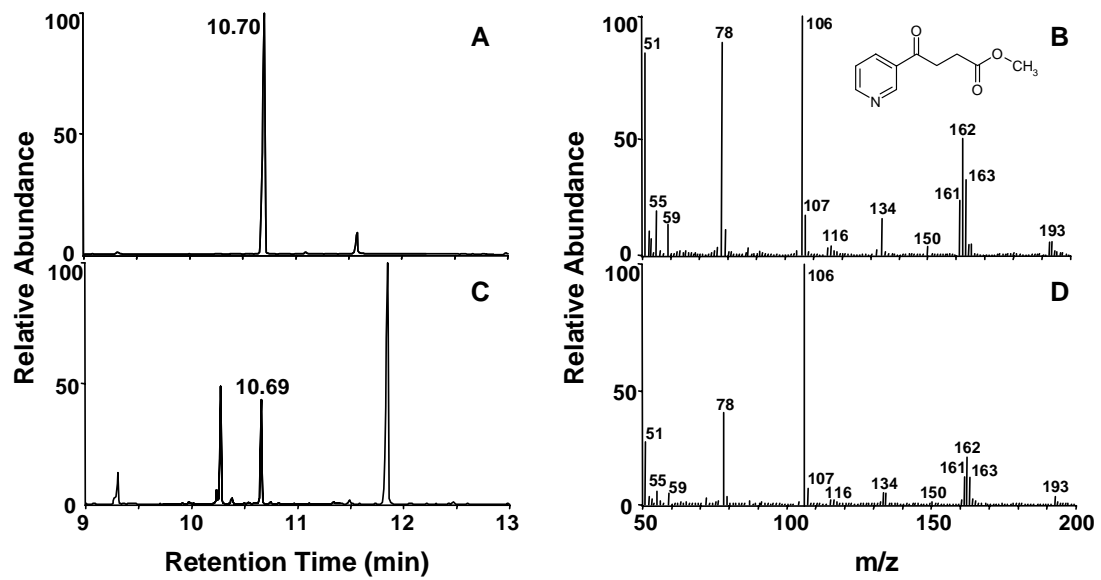


Figure 5:

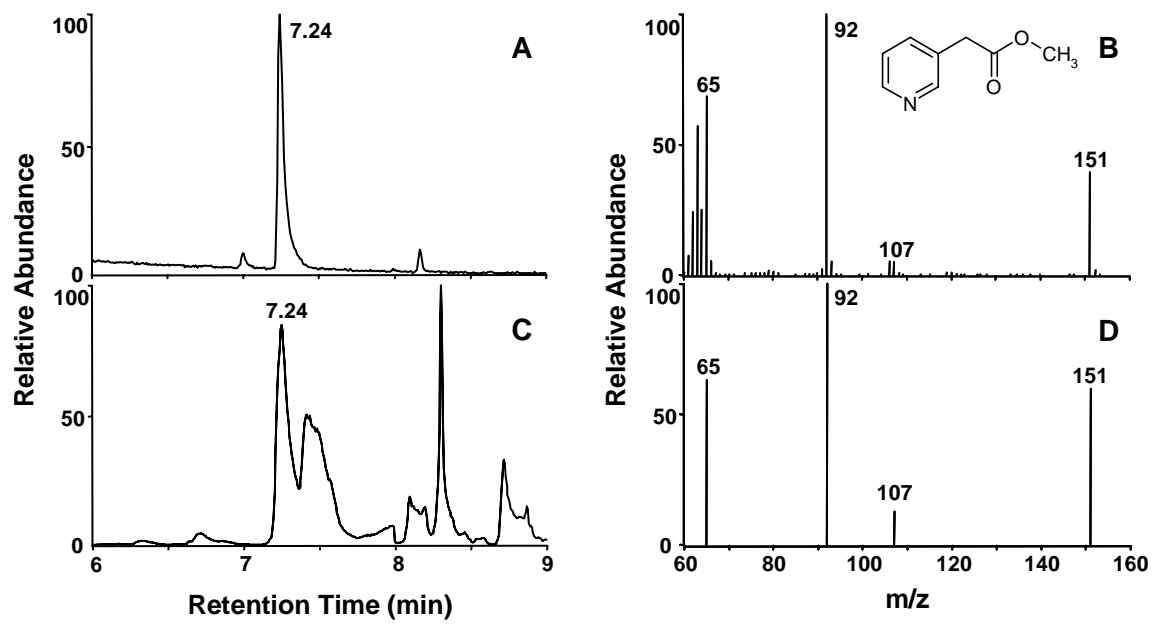
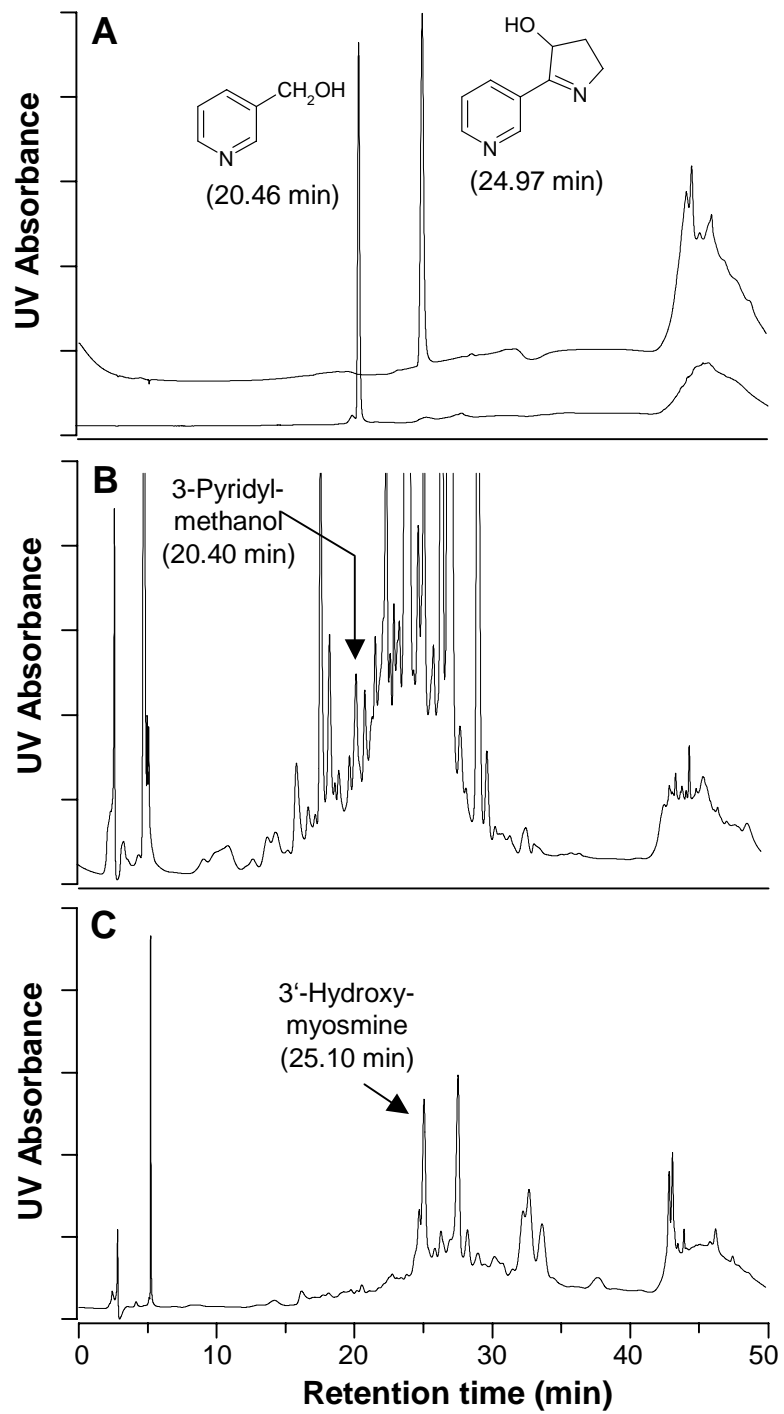


Figure 6:

Figure 7:



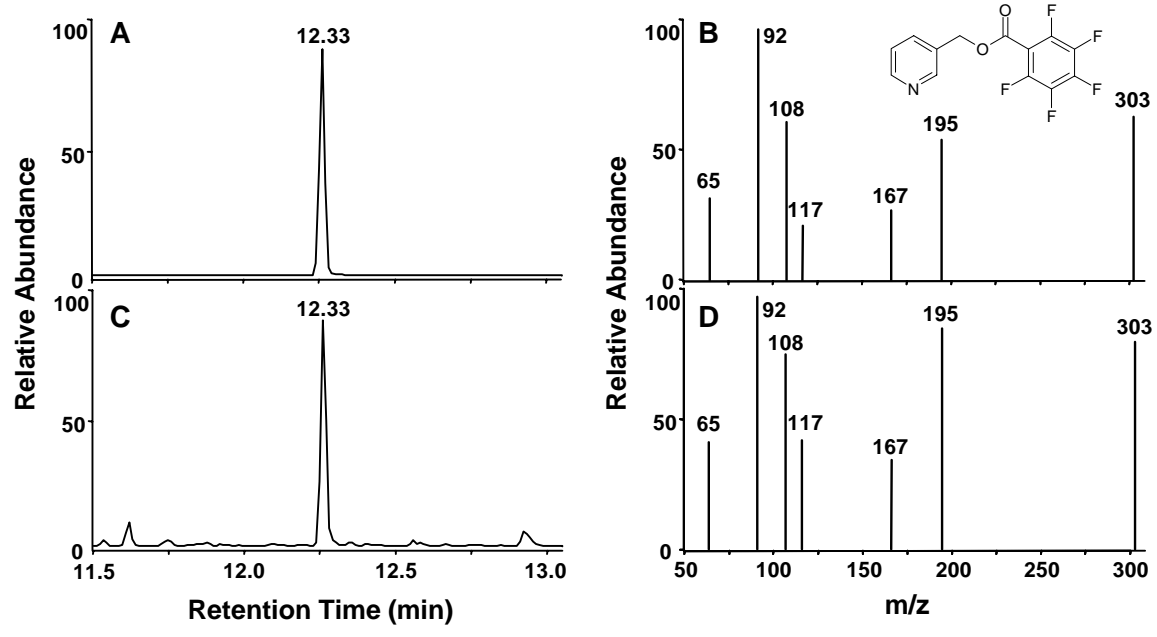


Figure 8:

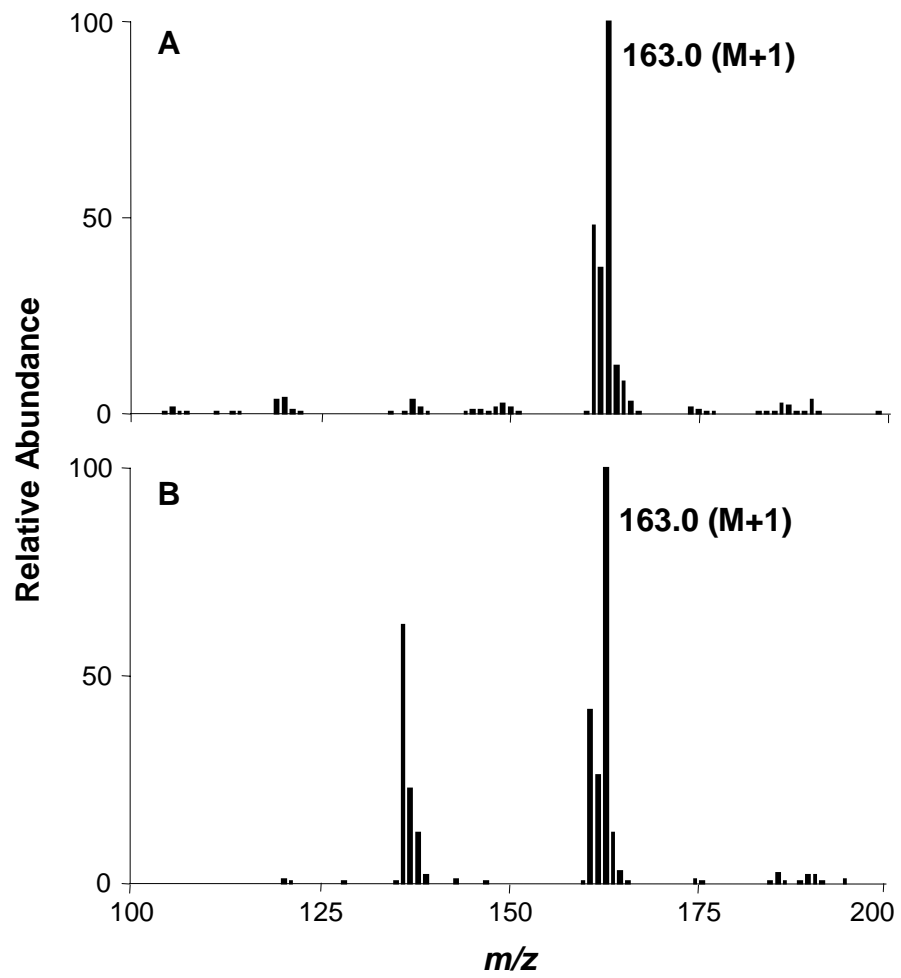


Figure 9: