METABOLISM OF MYOSMINE IN WISTAR RATS

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ABSTRACT:

The alkaloid myosmine is present not only 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'-nitrosornicotine. 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 μmol/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 μmol/kg, a higher percentage of the radioactivity was excreted in urine (86.2 ± 4.9% and 88.9 ± 1.7%) as compared with the higher doses, 0.5 and 50 μmol/kg, where only 77.8 ± 7.3% and 75.4 ± 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 (20–26%) and 4-oxo-4-(3-pyridyl)butyric acid (keto acid, 50–63%) using UV-diode array detection and gas chromatography-mass spectrometry 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 a 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 to 300 μg/plain nonfilter cigarette (Baker, 1999). Only small attempts were made to evaluate its possible toxicological relevance besides early investigations about nicotineoids 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 that from exposure to environmental tobacco smoke.

In the course of the first studies on the metabolism of the tobacco-specific nitrosamines (TSNAs), 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosornicotine (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 the 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) (Fig. 1). The formation of HPB occurs presumably through a 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 NNNK after metabolic activation by N-hydroxylation (Hecht, 1998, 2003). In the presence of nitrite, nitrosation of myosmine in human gastric juice yielded NNN and HPB with optimal reaction conditions at pH 2 to 5 (Zwickenpflug, 2000; Wilp et al., 2002). Preliminary studies on the peroxidation of myosmine yielded keto acid, 3-pyridylmethanol, HPB, and normicotyline 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"/pol", 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 to 5, covalent binding of radioactivity derived from 3H-myos-
mine 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 were 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 be seen not only as a 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.

**Materials and Methods**

**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).

**Synthesis of Myosmine, 3'-Hydroxymyosmine, Keto Acid, and HPB.** Myosmine (HPLC purity >99%) and keto acid (HPLC purity >98%) were synthesized according to the method of Brandage 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 with the method of 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 (Borchern, 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 (sniff R; sniff, Soest, Germany) and water were provided ad libitum. For collection of urine and feces, the rats were housed individually in stainless steel metabolism cages. After 24 h of adaptation, exposure to myosmine was managed by feeding groups of 4 rats/dose contaminated apple bites. Each bite was prepared with 2 pmol of [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 at 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) 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 μl of Ultima Gold XR scintillator (PerkinElmer Life and Analytical Sciences, Frankfurt/Main, Germany) for determination of total radioactivity in a Tri Carb 2500 TR Liquid Scintillation Analyzer (PerkinElmer Life and Analytical Sciences). Feces were mixed with 2.5 ml of methanol/g of wet weight and homogenized with an Ultra-Turrax (Bachofer, Reutlingen, Germany). To 0.1 g of the homogenates 0.2 ml of hydrogen peroxide and 0.2 ml of perchloric acid were added and incubated at 80°C for 30 min. After cooling, 10 μl of liquid scintillator was added to the samples, which were measured after overnight storage in the dark.

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

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**FIG. 1.** In vitro nitrosation of myosmine and identified metabolites in rat urine after treatment with [pyridine-5-3H]myosmine.
Derivatization of 3-Pyridylacetic Acid Standard, Keto Acid Standard, and a Urine Fraction. To 0.01 mmol (1.37 mg) of 3-pyridylacetic acid dissolved in 5 ml of acetonitrile, 0.03 mmol (4.15 mg) of potassium carbonate was added. After stirring the reaction mixture at room temperature for 10 min, 0.03 mmol (4.26 mg) of CH₃I was added. The reaction mixture was stirred under reflux for another 3 h. After removal of the acetonitrile 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 of acetonitrile. 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 of CH₃CN/H₂O (2:98 v/v) containing keto acid were concentrated under reduced pressure and derivatized as described above. The reaction mixture containing methylated 3-pyridylacetic acid was further cleaned up by thin-layer chromatography (TLC) on a analytical Kieselgel F254 plate (Merck) using CHCl₃/MeOH (10:1 v/v). The spot with Rₖ 0.7 was removed from the plate and eluted with CHCl₃/MeOH (3:2 v/v).

Derivatization of 3-Pyridylmethanol Standard and of a Urine Fraction. To 3-pyridylmethanol standard, 1 ml of NMe₂/CH₃Cl₂ (1:1 v/v) and 10 μl of a pentafluorobenzoyl chloride solution [10 μl of PFBCl and 490 μl of NMe₂/CH₃Cl₂ (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 μl) was added and centrifuged at 2000g for 10 min.

An aliquot of a urine fraction [CH₃CN/H₂O (4:96, v/v)] was cleaned up by two-dimensional TLC on an analytical Kieselgel F254 plate (Merck) using CHCl₃/MeOH/NH₄OH (85:15:2, v/v). The spot with Rₖ 0.85:15:2, v/v) followed by CHCl₃/MeOH (3:2 v/v). The region with Rₖ values corresponding to 3-pyridylmethanol standard was removed from the plate and eluted with CHCl₃/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 CH₃CN/H₂O (8:92 v/v). An aliquot of the fraction was transferred on an analytical Kieselgel F254 plate (CHCl₃/MeOH/NH₄OH; 85:15:2, v/v). The spot with Rₖ 0.46 was removed from the plate and extracted with CHCl₃/MeOH (3:2 v/v). After removal of the solvent, the residue was further analyzed by LC/MS.

**HPLC Measurements.** Urine samples of 400 μl were filtered by centrifugation at 2200g through a 0.2-μm Nanosep MF filter (Pall, Dreieich, Germany) and analyzed by HPLC with online radioactivity monitoring and a scintillator pump (Ramona 2000; Raytest, Straubenhardt, Germany) adding 3 μCi of [1,125I]-labeled 4-Oxo-4-(3-pyridyl)butyric acid (0.015 mmol, 2.69 mg) was dissolved in 5 ml of acetonitrile, 0.03 mmol (4.15 mg) of potassium carbonate was added. After stirring the reaction mixture for 10 min at 1% CH₃CN/99% buffer, CH₃CN 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).

**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 ZBS50 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 (Agilent, Böblingen, Germany) coupled with a Sciex API 2000 (Applied Biosystems, Darmstadt, Germany). The HPLC apparatus 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 ± 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 administering myosmine 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 CHCl₃/MeOH (3:2, v/v) resulted in total recovery of myosmine to ensure that a defined amount of myosmine is picked up. The relative amounts of total radioactivity excreted in urine and feces (Table 1). The spread occurrence of myosmine in food. Extraction of a contaminated apple bite with CHCl₃/MeOH (3:2, v/v) resulted in total recovery of myosmine to ensure that a defined amount of myosmine is picked up.

Elimination of Myosmine. Between 87.9 ± 21.1 and 100.5 ± 26.8% of the radioactive dose was recovered in urine and feces (Table 1). 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 ± 4.9% and 88.9 ± 1.7%, was recovered in urine as compared with the two higher doses, where only 77.8 ± 7.3% and 75.4 ± 6.6% appeared in the urine.

Within the first 6 h after myosmine administration, 88.5 ± 2.5% (0.001 μmol/kg), 87.3 ± 7.6% (0.005 μmol/kg), 79.0 ± 22.1% (0.5 μmol/kg) and 78.1 ± 7.9% (50 μmol/kg) of the total radioactivity excreted in urine was recovered. After 24 h, 97.4 ± 1.4% (0.001 μmol/kg), 98.1 ± 0.4% (0.005 μmol/kg), 97.3 ± 1.1% (0.5 μmol/kg), and 95.2 ± 6.3% (50 μmol/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 Fig. 2. Besides two major peaks, three minor metabolites of myosamine were observed corresponding to 84 to 91% of total radioactivity recovered from HPLC. No unmetabolized myosamine 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, nornicotine, or NNN-N-oxide. The two major myosamine metabolites could be identified (see below) as 3-PAA and keto acid (Fig. 1). 3-PAA contributed to a similar percentage to the sum of all urinary metabolites, averaging 23.2 ± 2.6% over the whole concentration range (Table 1). In contrast, the percentage of keto acid increased significantly at the highest dose (63.1 ± 2.9%) compared with the three lower doses (52.0 ± 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 1; Fig. 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. On reducing the pH further to search for stronger acids, the chromatography became irreproducible. The main metabolite was identified as the keto acid by coelution with a standard (see Fig. 3 for correlation of UV-DAD and radio-HPLC retention times). Comparison of the UV spectra confirmed the assignment (Fig. 4). The last evidence was obtained by GC/MS measurements after converting the keto acid to its methyl ester (Fig. 5).

Attempts to identify the second largest signal headed toward 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 coeluted with the corresponding peak in HPLC (see Fig. 4 for UV-DAD 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 breakthrough fluid, and most of the substances interfering in the later GC/MS analysis were retained at the C₁₈ material. After methylation and further cleanup by TLC, GC-chromatograms and MS spectra demonstrated the presence of 3-pyridylacetic acid methyl ester (Fig. 6).

The urine fraction received after elution of the solid-phase extraction tube with CH₆CN/H₂O 4:96 contained 3-pyridylmethanol. Identification is based on coelution with a standard on HPLC (Fig. 7), UV spectra (Fig. 4), and GC/MS data after derivatization with pentafluorobenzoyl chloride (Fig. 8). 3'-Hydroxymyosmine was present in the urine fraction eluted with CH₆CN/H₂O 8:92 and was identified by HPLC-UV (Figs. 4 and 7) and LC/MS (Fig. 9). The identity of HPB was verified by coelution with an unlabeled standard and comparison of the UV spectra (Fig. 4; for correlation of HPLC-UV and radio-HPLC retention times, see Fig. 3).

Fig. 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.
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 the oral cavity and esophageal tract. Myosmine given to rats over a dose range of 0.001 to 50 μmol/kg body weight undergoes rapid and complete metabolism. The absence of unmetabolized 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 (Beckett et al., 1971; Schepers et al., 1993; Hukkanen et al., 2005). Apart from 3′-hydroxymyosmine, the identified metabolites in rat urine (Fig. 1) are in part well known from metabolism studies with other tobacco nicotinoids and TSNAIs. HPB and keto acid are metabolites of NNK, NNN, and nicotine. 3-PAA was identified as a urinary metabolite in animals or humans after administration of nicotine, cotinine, and keto acid (McKennis et al., 1964a,b; 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, or metabolism in different organs or tissues, but mainly to the physicochemical 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-butoenoic 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 still remains speculative (Hukkanen et al., 2005). Studies on the metabolism of (E)-metanicotine pointed out 4-(3-pyridyl)-3-butoenoic acid as a major and 3-PAA as a terminal metabolite (Benchéri 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 a metabolite of NNN and NNK, although keto acid and hydroxy acid are the major urinary metabolites of these two TSNAs 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 TSNAs (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 (Hecht et al., 1981; McIntee and Hecht, 2000). 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 unmetabolized (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.

The contribution of myosmine nitrosation to HPB formation in rats must be discussed and differentiated. Although no NNN was found in rat urine and, therefore, in vivo nitrosation seems to contribute only a little to myosmine metabolism, HPB and its postulated oxidation product keto acid were detected in rat urine, accounting for 52 to 66% of total radioactivity recovered from HPLC. Another activation pathway besides 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 Zwickenpflug, 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 unstable 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 a 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


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