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N-Methylpyridoxamine: Novel canine vitamin B_6 urine metabolite

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Abstract—Cation-exchange HPLC analysis of urine from dogs given large daily doses of pyridoxamine revealed an unidentified metabolite hypothesized to be *N*-methylpyridoxamine. Identity was established by *N*-methylpyridoxamine synthesis and HPLC comparison to the canine metabolite. Compound synthesis was confirmed by IR, NMR, UV–vis and emission spectroscopy. It seems to have less fluorescent character than other routinely-measured vitamin B_6 metabolites. Upon administration of substantial pyridoxamine doses, *N*-methylpyridoxamine appears to be a quantifiable canine urine metabolite, although, at either pharmacological or dietary pyridoxamine intakes, its relevance to vitamin B_6 metabolism in other species, including humans, is not yet determined.

Vitamin B_6, a family of related metabolically interconverted molecules (vitamers), is utilized in over 100 biochemical reactions involved in glucose, lipid, and amino acid metabolism, production of proteins and genetic material, and its deficiency may affect immune responses and tumor growth. While seven major metabolites are well known (pyridoxal, pyridoxine, pyridoxamine, the phosphorylated forms of these three, and 4′-pyridoxic acid), pyridoxal 5′-phosphate (PLP) is the biologically active form. In humans urinary 4′-pyridoxic acid (PA) accounts for approximately 40–60% of ingested vitamin B_6, with other unidentified metabolites having been reported.[1] By contrast, PA is a minor urinary metabolite in domestic felines and canines.[2] As significant species dissimilarities appear to exist in overall vitamin B_6 metabolism, finding an appropriate animal model for human vitamin B_6 metabolism remains unresolved.[1f,2a]

One vitamer, pyridoxamine (PM, Pyridorin™), administered pharmacologically, is reported to inhibit conversion of Amadori ketoamine products into advanced glycation end products (AGEs) produced in a variety of conditions (including aging, atherosclerosis, and Alzheimer disease) and significantly contribute to development of type I and II diabetic complications. PM inhibition may lower accumulation of AGEs in collagen-rich tissues where complications, such as nephropathy, renal disease, cardiopathy, neuropathy, and retinopathy occur.[3] PM apparently prevents glycated protein breakdown into AGEs and reduces urine levels of transforming growth factor-β (specifically TGF-B1), which stimulate matrix production where AGEs may be found. Pyridoxamine may have antioxidant activity, and may trap reactive intermediates, which could prevent lysine residue modification by advanced lipoxidation products (ALEs) during lipid peroxidation. These effects are reported at pharmacological PM concentrations several thousand times physiological concentrations, which, depending upon organism and tissue, range from 0.001 (rat plasma) to 15 μM (rat kidney).[3b,c] Utilizing PM to control complication development is still being studied, but it repeatedly has been shown in clinical studies to be safe, effective, and well-tolerated. No toxicity has been reported in animal or clinical studies to date.[3a,c,4]

In an animal study investigating toxicity effects of pyridoxamine,[5] an unusual metabolite was detected by cation-exchange HPLC in urine from dogs given large daily dosages of pyridoxamine (100 mg/kg) for approximately four weeks (in conjunction with Harlan Teklad

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9682 Dog Maintenance diet). It was hypothesized to be *N*-methylpyridoxamine, based upon similarities to *N*-methylpyridoxine found in urine of felines given pyridoxine.[1f] To confirm this metabolite's identity, *N*-methylpyridoxamine was synthesized and determined to have the same cation-exchange HPLC characteristics as the urine metabolite.

In vivo N-methylation of aromatic nitrogen heterocycles varies among species, generally being higher in cats, guinea-pigs, gerbils, rabbits, and hamsters than in rats, mice or humans. The presence of "pyridine *N*-methyltransferases" in the cytosol of dialyzed rabbit lung and liver tissue appears to utilize *S*-adenosyl-L-methionine (SAM) as the methyl donor.[6] This methyltransferase activity may extend to vitamin B_6 forms, although the pathway by which these are methylated is not yet known. Another essential nutrient, niacin, required for synthesis of metabolic nicotinamide coenzymes, is normally excreted as a variety of methylated (also utilizing SAM as the methyl donor) and hydroxylated products, although untransformed niacin vitamers can be found in the urine as dosages increase.[7] This is in contrast to vitamin B_6, which appears to be methylated in response to high concentrations.[1f] This methylation process, at high doses of PM, may be an alternative path (in dogs and possibly other species) when other metabolic processes become saturated. Other pathways producing additional metabolites may also be possible; after a high dose of pyridoxamine, one study reported that only 31% of that dose could be recovered from human urine as pyridoxal, pyridoxamine, pyridoxine, or 4-pyridoxic acid.[1g]

Detection of urinary vitamin B_6 metabolites used published cation-exchange HPLC protocols.[8] This metabolite was detected when the final gradient step was extended 85 min. Chlorite post-column derivatization of samples was utilized, but it was determined that derivatization did not alter detection.

N-methylpyridoxamine dihydrochloride was synthesized (Fig. 1) using modifications of published procedures for methylating vitamin B_6 derivatives.[9–11,13] Taking advantage of the significant acidity difference of pyridinium and exocyclic ammonium ions, a proton protecting group was used for pyridoxamine's primary amine, similar to the recently described procedure for polyamines.[14] Our synthesis from pyridoxamine dihydrochloride, which was not optimized, involves two steps and gives a comparable yield (12.9%) to Pocker and Fischer's five step synthesis from pyridoxal hydrochloride (16.3%).[9b]

Pyridoxamine derivatives are reported to be photosensitive and thermally unstable.[9b] To avoid decomposition, exposure to light was minimized and, whenever possible, vitamin B_6 derivatives were not heated above 45 °C. Otherwise, pyridoxamine hydrochloride and *N*-methylpyridoxamine dihydrochloride salts were stable indefinitely in solid state and in solution. *N*-methylpyridoxamine was stable overnight in aqueous 1.0 M NaOH solutions. However, the zwitterion appears to be unstable, as attempts to isolate it failed. Addition of one equivalent of silver carbonate to an aqueous salt solution, followed by filtration of silver salts and removal of water under vacuum, led to an unidentified dark residue. Addition of more than two equivalents aqueous ammonia also led to decomposition, even in solution.

A 73 μM solution of synthesized *N*-methylpyridoxamine (molar mass = 255.13 g/mol) in 0.02 N HCl (pH = 1.0) had a single 297 nm UV–vis peak (scanned 200 nm to 500 nm). In 0.02 N NaH_2PO_4 buffer (pH = 7.0) this same concentration revealed two peaks: 254 nm, and a more intense peak at 331 nm. In 0.5 M sodium phosphate buffer, pH 6.3, synthesized *N*-methylpyridoxamine had emission maximum at 373 nm and excitation maximum at 331 nm. Comparatively, pyridoxamine has emission maximum at 392 nm and excitation maximum at 324 nm.

This metabolite eluted from cation-exchange HPLC after normally detected vitamin B_6 metabolites, suggesting it was more positively charged than pyridoxamine (last of normally eluting metabolites) eluting at pH 5.9. Increasing the pH of the final eluent buffer to 6.3 decreased the metabolite's elution time by approximately 20%. This metabolite was not detected in urine of unsupplemented canines; however, spiking control urine with synthesized compound gave the same cation-exchange HPLC peak as supplemented urine. Adding synthesized *N*-methylpyridoxamine to the supplemented canine urine revealed no additional peaks.

The detection limit for *N*-methylpyridoxamine is 24 nmol/L sample (or 1.5 ng of injected sample), compared to detection limits for PM (2.3 nmol/L) and PA (1.4 nmol/L) and PLP (1.6 nmol/L) with this HPLC method utilizing the chlorite derivatization method.[8a] It has not been determined if *N*-methylpyridoxamine is

Table 1. Physical characteristics—*N*-methylpyridoxamine dihydrochloride

N-Methylpyridoxamine dihydrochloride (molar mass = 255.13 g· mol^{-1})		
pH	Ultraviolet maxima (nm)	Calculated ε (M^{-1} cm^{-1})
1	297	6722
7	331	7641

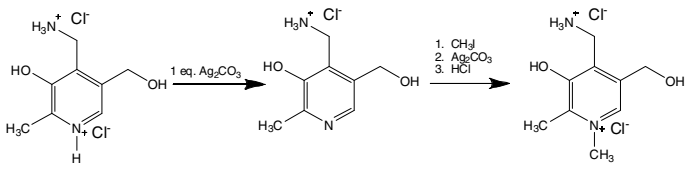

Figure 1. Preparation of *N*-methylpyridoxamine dihydrochloride.

a metabolite in dogs or other species without profound supplementation.

Based upon peak areas in these supplemented canine urines, pyridoxamine initially appeared to be the major metabolite (94%), with *N*-methylpyridoxamine as the second metabolite (3%). However, with standards with equal concentrations of *N*-methylpyridoxamine and pyridoxamine, *N*-methylpyridoxamine fluoresced approximately one-tenth as strongly as pyridoxamine (excitation wavelength 330 nm, detection wavelength 420 nm); correcting for this response factor, it appears that *N*-methylpyridoxamine represents approximately 10%, with pyridoxamine representing close to 87%, of the excreted metabolites in these supplemented dogs.

Ultraviolet spectra of the synthesized compound taken at pH 1 and pH 7 determined absorption maxima and molar extinction coefficients (ε) at two different pH levels (Table 1). Alterations in these maxima and extinction coefficients based upon pH are well known.[15] Synthesized *N*-methylpyridoxamine dihydrochloride gave strong absorption peaks at 254 and 331 nm at pH 7.0. Strong absorption peaks were noted at 294 and 336 nm (pH 7) for a similar compound, 1-methyl-3-hydroxy-4-amino-methylpyridinium chloride, and both peaks are reported to be $\pi - \pi^*$ transitions.[16]

In conclusion, we present *N*-methylpyridoxamine as a newly recognized vitamin B_6 metabolite in dogs heavily supplemented with pyridoxamine, with identity confirmed by unambiguous synthesis. It appears to have less inherent fluorescence than other detected B_6 vitamers at wavelengths routinely utilized for urine vitamin B_6 cation-exchange HPLC assays with fluorescent excitation and detection. The ability to now identify this metabolite may lead to its detection in other species as well, providing increased understanding of influences on vitamin B_6 metabolism.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.02.013.

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10. Infrared spectra were obtained on Nicolet 550 FTIR. NMR spectra (^{1}H and ^{13}C) were obtained on Varian Mercury VX spectrometer 200 MHz. Decomposition points were obtained on Thomas-Hoover melting point apparatus. Ultraviolet–visible spectra were performed on a Shimadzu model 160 spectrometer, and fluorescent spectra were obtained on Fluorolog TAU-3.

11. Pyridoxamine hydrochloride was synthesized by adding 2.92 g (10.6 mmol) silver carbonate[12] to 5.03 g (20.9 mmol) pyridoxamine dihydrochloride in 7.6 mL of water. After vigorous gas evolution, the tan slurry was stirred 2 h at ambient temperature. The slurry was collected by vacuum filtration, and the gray filter cake was washed with water, which was removed under vacuum from the brown filtrate, leaving 3.90 g of light tan solid (91.1% yield): dec. 244 °C. IR (KBr) 3334, 3059, 2809, 2087, 1532, 1476, 1434, 1388, 1306, 1307, 1221, and 1132 cm^{-1}.[1] ^1H NMR (DMSO-d_6) 7.94 (singlet, 1H), 4.58 (singlet, 2H), 4.08 (singlet, 2H), and 2.42 (3H). Due to significant degree of proton exchange, N–H and O–H protons were not readily observed. However, all absorptions are shifted relative to the dihydrochloride. ^{13}C NMR (DMSO-d_6) 150.7, 146.6, 138.4, 134.8, 127.9, 59.0, 33.8, 19.5.
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13. Pyridoxamine hydrochloride (3.00 g, 14.6 mmol) was placed into a three-neck 3000 mL round-bottom flask with 1100 mL benzene and 240 mL methanol. The cloudy mixture was heated at reflux overnight. After 24 h reflux, an additional 90 mL of methanol and 35 mL (560 mmol) methyl iodide were added to the still-cloudy mixture. After reflux for 21.5 h, the cloudy mixture had become a clear solution. 20 ml. (320 mmol) of methyl iodide was added in two portions while the reflux was continued for another 55 h. The solution was reduced to one-third volume, and a red oil, which separated from the solvent, formed a yellow solid upon stirring. The solid, collected by vacuum filtration, was washed with diethyl ether; 5.023 g of crude product was obtained. Silver carbonate (2.00 g, 7.25 mmol) was added to 2.52 g of crude yellow solid in 50 mL water with gas evolution. After stirring for 30 min, the slurry was collected by vacuum filtration. The light yellow-green filter cake was washed with water. 12.1 mL concentrated hydrochloric acid was added to the light orange filtrate, and water was removed under vacuum. Upon recrystallization from absolute ethanol, 0.7613 g of tan solid was obtained. ^1H and ^{13}C NMR in DMSO-d_6 indicated the solid was a mixture of *N*-methylpyridoxamine dihydrochloride and pyridoxamine dihydrochloride starting material. Product was isolated from 0.179 g of this mixture on anionic exchange column (10.3 cm × 1 cm, Dowex 1-X8, 100–200 mesh). After acidifying with 1.0 M HCl, fractions containing product (identified by UV) were combined, and water was removed under vacuum. The resulting white solid was extracted with absolute ethanol. A tan solid (0.0628 g; 14.2%) was obtained upon removal of ethanol under vacuum. The stated yield takes into account that only portions of crude *N*-methylpyridoxamine salt and crude recrystallized *N*-methylpyridoxamine dihydrochloride were used to complete the synthesis. dec. 226 °C. IR (KBr) 3192, 2921, 1467, 1426, 1386, 1211, and 1050 cm^{-1}. ^1H NMR (D_2O) δ = 8.35 (singlet, 1H), 4.37 (singlet, 2H), 4.20 (singlet, 3H), and 2.67 (singlet, 3H). The hydroxy methylene protons are obscured by the HOD peak at 4.75 ppm. ^{13}C NMR (D_2O) 154.4, 146.2, 137.6, 135.4, 58.8, 46.7, and 13.9. ^1H NMR (DMSO-d_6) 8.58 (singlet, 1H), 4.80 (singlet, 2H), 4.27 (singlet, 3H), 4.19 (singlet 2H), and 2.42 (singlet, 3H). ^{13}C NMR (DMSO-d_6) δ = 153.7, 145.5, 139.2, 135.5, 135.4, 58.0, 46.4, 33.3, 14.1.
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