Identification of 5-pyridoxic acid and 5-pyridoxic acid lactone as metabolites of vitamin B_6 in humans

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Plasma and urine from individuals ingesting large amounts of vitamin B_6 contain several unidentified vitamin B_6 metabolites. Based on movement in 6 TLC solvents, elution in HPLC, and ultraviolet and infrared spectra, two of these metabolites have been identified as 5-pyridoxic acid and its lactone. These two compounds can account for 10–20% of the urinary vitamin B_6 metabolites.

Keywords: vitamin B_6; 5-pyridoxic acid; human

Introduction

The major urinary metabolite of vitamin B_6 in many mammalian species is 4-pyridoxic acid. 4-Pyridoxic acid lactone, pyridoxine, and pyridoxal may also be found, particularly at high vitamin B_6 intakes. In addition to these common metabolites, the existence of unidentified metabolites of vitamin B_6 has been recognized for many years. Using ^{14}C-pyridoxine, Tillotson et al.[1] found at least nine unidentified vitamin B_6 metabolites in human urine. We have identified pyridoxal-3-sulfate, pyridoxine-3-sulfate and *N*-methylpyridoxine in cats.[2] We also identified 3-sulfate esters of 4′- and 5′-deoxypyridoxine in some species and oxidation of the 5′-position of 4′-deoxypyridoxine in guinea pigs, rabbits, swine, primates, and humans.[3,4]

Edwards et al.[5] reported two unidentified peaks in the plasma of subjects with hyperoxaluria being treated with 1.9 mmol (400 mg) pyridoxine hydrochloride or more per day. Normal subjects ingesting similar doses showed similar metabolites. One was postulated recently as 4-pyridoxic acid lactone.[6] The other was postulated to be isopyridoxal but found not to be. We had observed several unidentified peaks in addition to 4-pyridoxic acid lactone in the urine of subjects ingesting 0.97 mmol (200 mg) or more of vitamin B_6/day. We now present evidence that two of these peaks are 5-pyridoxic acid and its lactone. The lactone has been synthesized previously,[7,8] and both the free acid and lactone have been studied in connection with microbial metabolism of vitamin B_6.[9-12]

Materials and methods

Synthesis

The synthesis of 5-pyridoxic acid lactone was based on the method of Korytnyk et al.[8] Isopropylidenepyridoxine[13] (4.07 mmol) was dissolved in 20 mL water and adjusted to pH 3.9. Potassium permanganate (7.0 mmol) dissolved in 20 mL water was added dropwise over 10 min. The mixture was adjusted to pH 7.3 with sodium hydroxide, stirred for 15 min, and then centrifuged. The yellow supernatant was concentrated to 5 mL volume. The pH was adjusted to 5.0 with hydrochloric acid (10 mol/L), resulting in a white precipitate. The suspension was centrifuged and the solid was washed with ethanol. After resuspension in 12 mL water, 0.4 mL of hydrochloric acid (10 mol/L) was added to make a solution. This was heated on a steam bath for 60 min, then evaporated to dryness. The solid was suspended in ethanol. The centrifuged solid and the crystallized product from concentration of the ethanol supernatant were combined and washed with ethyl ether and dried. The final product was 5-pyridoxic acid lactone hydrochloride. The yield was 2.1 mmol (430

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mg) (52%) mp 245–250° C dec (literature[10] 242–246° C dec).

Metabolite isolation

Metabolites were isolated from the urine of a subject who had been ingesting 4.9 mmol (823 mg) pyridoxine daily for about a year in an effort to treat swelling in her hands. The sample contained about 40 mg total of unknown metabolites in 140 mL. The sample was adjusted to pH 3 and applied to a Dowex 50W–X4 100–200 mesh cation exchange column, 2 × 25 cm in the ammonium form. Fractions were eluted using ammonium acetate (0.1 mol/L pH 5.0). Fractions containing the desired metabolites were combined and applied to the Dowex column twice more. An additional application to Dowex column was made and the metabolites eluted in ammonium hydroxide (0.18 mol/L). The desired fractions were identified by HPLC, combined and concentrated under vacuum at 35° C–40° C. The resulting solution contained both 5-pyridoxic acid and the lactone. Therefore, after adjustment to pH 1 with hydrochloric acid, the sample was heated in a boiling water bath for 15 min to lactonize the entire sample. Sample was concentrated to 1.5 mL and the pH was adjusted to 4.0 with ammonium hydroxide (3 mol/L) to precipitate lactone. The centrifuged solid was washed with acetone followed by ethyl ether and then dried.

Traces of ammonium chloride salt had to be removed to obtain a satisfactory infrared spectrum. This was done by suspending the solid sample in several mL water and adjusting the pH to 11.2 with sodium hydroxide. Concentration under vacuum eliminated the ammonia. The sample was acidified to pH 1 and heated again to lactonize any free acid. The sample was evaporated to dryness and the solid was washed with acetone followed by ethyl ether.

Urine samples were also collected from four normal, male volunteers after a single 0.97 mmol (200 mg) oral dose of pyridoxine hydrochloride. Milk samples were collected after a similar dose from a non-nursing woman who had given birth recently.

Cation exchange HPLC[14,15] was used to analyze samples. The gradient program routinely used for total B_6 analysis was expanded to allow resolution of the new metabolites from the other B_6 vitamers. The gradient was programmed as follows: 0–10 min 100% Solvent A, 10–20 min 100% A to 100% B, 20–25 min 100% B, 25–35 min 100% B to 60% B/40% C, then to 100% C at 37 min and 100% C until 45 min. Adjustment of effluent to pH 7 using post column reagent is especially crucial to ensure detection of 5-pyridoxic acid and its lactone.

Ascending thin-layer chromatography was performed on silica gel type H on glass plates prepared in our laboratory with visualization by UV light. Ultraviolet spectra were performed on a Shimadzu UV-160 recording spectrophotometer. Infrared analyses were performed using KBr pellets on a Beckman Acculab 3.

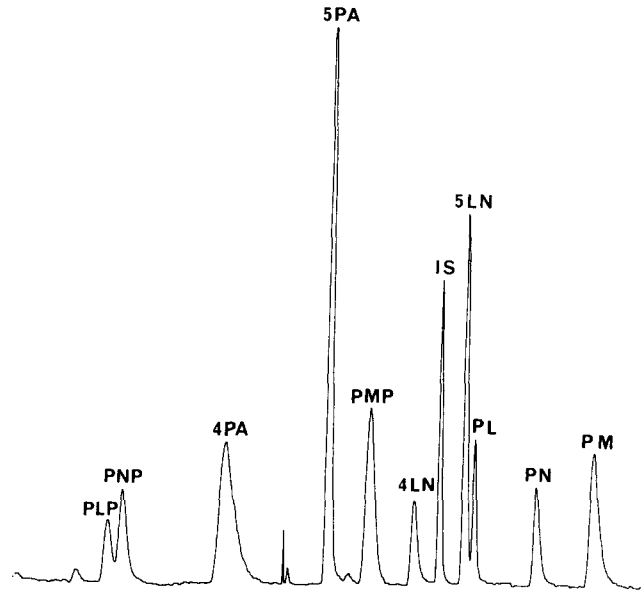

Figure 1 Chromatogram of a standard mixture of vitamin B_6 metabolites. Amounts injected (ng) and elution times (min) were: pyridoxal 5′-phosphate (PLP) 10.2, 7.60; pyridoxine 5′-phosphate (PNP) 10.0, 8.75; 4-pyridoxic acid (4PA) 8.9, 16.85; 5-pyridoxic acid (5PA) 50, 24.95; pyridoxamine 5′-phosphate hydrochloride (PMP) 11.1, 28.3; 4-pyridoxic acid lactone hydrochloride (4LN) 20, 31.75; internal standard (IS) 50, 33.8; 5-pyridoxic acid lactone hydrochloride (5LN) 200, 35.75; pyridoxal hydrochloride (PL) 11.9, 36.55; pyridoxine hydrochloride (PN) 10.6, 41.45; and pyridoxamine dihydrochloride (PM) 10.6, 46.00.

Results

The large peak which eluted between 4-pyridoxic acid and pyridoxamine-5′-phosphate in our HPLC system was our main focus of attention (*Figure 1*). Fractions containing this peak were collected and re-chromatographed after base and acid hydrolysis in a boiling water bath. Base had no effect, but acid treatment converted the peak to a compound with an elution time similar to pyridoxal. This seemed logical since we had previously isolated acid-hydrolyzable conjugates of pyridoxal, pyridoxine, and deoxypyridoxine in other species.[3,4] Expansion of our usual gradient, however, indicated that the conversion product could be separated from pyridoxal and that urine samples from subjects on high B_6 intakes with apparent large pyridoxal peaks actually contained a mixture of pyridoxal and this conversion product. We discovered that this conversion product ran the same as our synthetic 5-pyridoxic acid lactone and that our synthetic lactone could be delactonized with base to a product (5-pyridoxic acid) which ran the same as the original metabolite. Furthermore, the acid-treated conversion product of the metabolite, which eluted with 5-pyridoxic acid lactone, could be delactonized with base back to a compound which eluted with 5-pyridoxic acid. Purified preparations of this metabolite in lactonized and delactonized form matched corresponding forms of the synthetic material in six different TLC solvent systems (*Table 1*). The UV spectra of the corresponding forms

Table 1 R_f of 5-pyridoxic acid and urinary metabolite on silica gel

	5-pyridoxic acid		Metabolite	
Solvent[a]	Lactonized	Delactonized	Lactonized	Delactonized
A	0.99	0.99	0.99	0.99
B	0.82	0.71	0.82	0.71
C	0.98	0.39	0.98	0.39
D	0.49	0.20	0.49	0.20
E	0.57	0.27	0.57	0.27
F	0.78	0.63	0.78	0.63

[a]A - 0.5% ammonium hydroxide; B - 95% ethanol; C - Chloroform:methanol (70:30); D - 2-butanol:6% ammonium hydroxide (25:8); E - Isoamyl alcohol:acetone:diethylamine:water (24:18:8:6); and F - 1-butanol:water:pyridine:acetic acid (30:24:20:6).

Table 2 Ultraviolet maxima (nm)

	pH 1	pH 7
5-pyridoxic acid		
Literature[9]	296	327
Synthetic	297	324
Metabolite	297	326
5-pyridoxic acid-lactone		
Literature[9]	252	277
	292	321
Synthetic	252	277
	290	320
Metabolite	252	275
	291	320

of the isolated and synthetic products matched each other and the values reported in the literature[9,10] (*Table 2*). The infrared spectra of synthetic 5-pyridoxic acid lactone and the lactonized metabolite were superimposable (*Figure 2*).

The relative occurrence of 5-pyridoxic acid and its lactone is highest in the first urine samples taken after large vitamin B_6 doses. In four subjects ingesting single doses of 200 mg of pyridoxine hydrochloride, urine samples taken 4 hr later contained 10–20% of the total measured B_6 excretion in those samples as 5-pyridoxic acid and its lactone, with the amount of 5-pyridoxic acid about twice as much as the lactone. In a subject ingesting 1000 mg pyridoxine hydrochloride daily, the 2 urines in a 24 hr period with the highest excretion amounts contained 11% and 16% of the total vitamin B_6 metabolites as 5-pyridoxic acid and its lactone. In these samples, the lactone was the predominate form; however, these samples had been stored much longer, 6 months instead of 1 month as the samples from the above 4 subjects were. Plasma analyses of 2 subjects (*Table 3*) indicate a much higher relative occurrence of 5-pyridoxic acid in the 4.9 mmol dose subject. Although the subject consuming 4.9 mmol vitamin B_6/day was convinced that her large vitamin B_6 intake was beneficial and did not perceive any ill effects, we informed her of the potential toxicity of such high doses and recommended that she confer with her physician about reducing her vitamin B_6 intake to less than 2.4 mmol (500 mg/d). In milk obtained 3 hr after a 0.97 mmol dose of pyridoxine hydrochloride, 5-pyridoxic acid accounted for 12% of the vitamin B_6 metabolites.

Discussion

The interconversion between 5-pyridoxic acid and its lactone appears to occur more readily than that between the 4-pyridoxic acid and its lactone. This was evident in isolation and purification steps where exposure for an hour or more at room temperature to

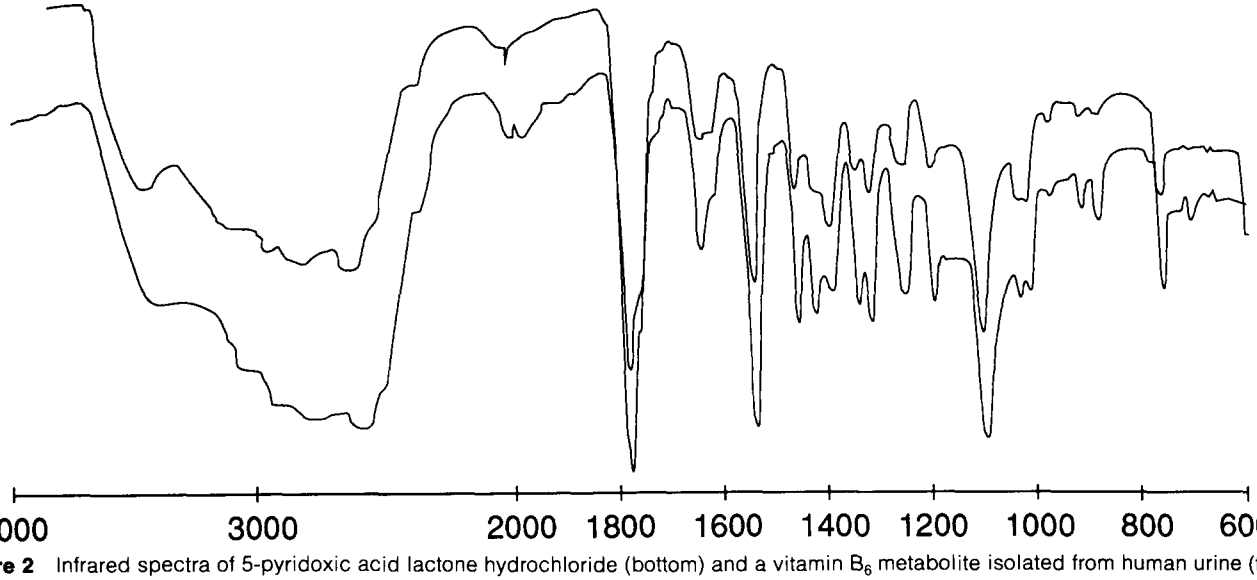

Figure 2 Infrared spectra of 5-pyridoxic acid lactone hydrochloride (bottom) and a vitamin B_6 metabolite isolated from human urine (top).

Table 3 Vitamin B_6 metabolites (nmol/L) in the plasma of two subjects receiving high vitamin B_6 intakes

Subject[a]	PLP[b]	4PA	5PA	PL	PN	Total
A	138	2588	292	4108	109	7235
B	318	4937	8488	14459	36735	64937

[a] Sample from subject A was obtained 4 hr after a single, oral dose of 0.97 mmol pyridoxine hydrochloride. Sample from subject B was obtained 3 hr after a 2.4 mmol dose in a woman who had been consuming 4.86 mmol/day for 6 mo.
[b] PLP = pyridoxal 5'-phosphate; 4PA = 4-pyridoxic acid; 5PA = 5-pyridoxic acid; PL = pyridoxal; PN = pyridoxine.

slightly acidic or basic pH could cause some conversion. We have observed that older urine samples have less 5-pyridoxic acid and more lactone than fresher ones.

While the presence of 5-pyridoxic acid and lactone is quite obvious in samples from subjects receiving 0.97 mmol (200 mg) or more of vitamin B_6, we are unsure whether they are excreted at all with lesser or normal intakes. Considering the smaller dilution of sample that would have to be used and the complexity of urine chromatograms, these peaks would be hard to identify and quantitate with confidence, without some type of purification of sample. Under the analysis conditions we use for HPLC, the 5-pyridoxic acid peak is about 25% of the area of the peak from a comparable quantity 4-pyridoxic acid. The 5-pyridoxic acid lactone is 8–10 times less fluorescent than the 5-pyridoxic acid, making its true quantity greater than its small peak size would seem to indicate. Presumably, as with 4-pyridoxic acid lactone, the fluorescence could be enhanced with a more basic post column reagent and altered set of wavelengths.

The identification and quantitation of 5-pyridoxic acid and lactone will help to account more completely for total B_6 excretion. In past high dose studies, we have never been able to account fully for B_6 intake on the basis of known B_6 excretion productions. A 10–20% increase in the measured metabolites in samples should help to bring total excretion measurements closer to intakes.

Previous studies of oxidation of the 5-position of pyridoxine have dealt primarily with microbial metabolism.[9-12] However, we found that 5'-oxidation of 4'-deoxypyridoxine occurred in guinea pigs, rabbits, swine, primates, and humans[3] and continued in germfree guinea pigs,[16] thus demonstrating that this reaction can occur in mammalian tissues, at least in guinea pigs. At this time, we have not identified the enzyme involved in this process. Neither have we completely ruled out the possible involvement of the intestinal flora in our human subjects.

The occurrence of significant amounts of 5-pyridoxic acid derivatives appears to be limited to vitamin B_6 intakes of 1 mmol or more/day. However, in recent years vitamin B_6 has received considerable publicity in the lay press and, as indicated by our subject who was purchasing 2.4 mmol (500 mg) tablets, such intakes are not unknown in the general population either with or without medical supervision. In an adult, an intake of 973 umol (200 mg)/day amounts to about 14 umol/kg body wt. Doses of this magnitude have been used in treatment of a variety of conditions including asthma,[17] Down's syndrome,[18] autism,[19] premenstrual syndrome,[20] and carpal tunnel syndrome.[21] After reviewing the literature, Bendich and Cohen[22] concluded that vitamin B_6 intakes of less than 2.4 mmol (500 mg)/d appear to be safe, while doses exceeding 4.86 mmol (1000 mg)/d appear to be a high risk. Further work will be needed to determine whether the metabolites reported here contribute to the positive and/or negative effects associated with high vitamin B_6 intakes.

References

1 Tillotson, J.A., Sauberlich, H.E., Baker, E.M., and Canham, J.E. (1966). Use of carbon-14 labeled vitamins in human nutrition studies:pyridoxine. *Proc. 7th Intl. Congr. Nutr.* **5,** 554–557

2 Coburn, S.P. and Mahuren, J.D. (1987). Identification of pyridoxine-3-sulfate, pyridoxal-3-sulfate and N-methyl pyridoxine as major urinary metabolites of vitamin B_6 in domestic cats. *J. Biol. Chem.* **262,** 2642–2644

3 Coburn, S.P. and Mahuren, J.D. (1979). Major urinary metabolites of 4'- and 5'-deoxypyridoxine in various species. *IRCS Med. Sci.* **7,** 556

4 Coburn, S.P. and Mahuren, J.D. (1976). In vivo metabolism of 4'-deoxypyridoxine in rat and man. *J. Biol. Chem.* **251,** 1646–1652

5 Edwards, P., Liu, P.K.S., and Rose, G.A. (1989). A simple liquid chromatographic method for measuring vitamin B_6 compounds in plasma. *Clin. Chem.* **35,** 241–245

6 Edwards, P., Liu, P.K.S., and Rose, G.A. (1990). Liquid chromatographic studies of vitamin B_6 metabolism in man. *Clin. Chim. Acta.* **190,** 67–80

7 Harris, S.A., Stiller, E.T., and Folkers, K. (1939). Structure of Vitamin B_6 II. *J. Am. Chem. Soc.* **61,** 1242–1244

8 Korytnyk, W., Kris, E.J., and Singh, R.P. (1964). Pyridoxine chemistry: Synthesis of isopyridoxal, 5-pyridoxic acid lactone, and their derivatives. *J. Org. Chem.* **29,** 574–579

9 Burg, R.W. (1970). The catabolism of vitamin B_6. *Meth. Enzymol.* **18A,** 634–658

10 Rodwell, V.W., Volcani, B.E., Ikawa, M., and Snell, E.E. (1958). Bacterial oxidation of vitamin B_6. *J. Biol. Chem.* **233,** 1548–1554

11 Lee, Y.C., Nelson, M.J., and Snell, E.E. (1986). Enzymes of vitamin B_6 degradation. Purification and properties of isopyridoxal dehydrogenase and 5-formyl-3-hydroxy-2-methylpyridoxine-4-carboxylic acid dehydrogenase. *J. Biol. Chem.* **261,** 15106–15111

12 Nelson, M.J.and Snell, E.E. (1986). Enzymes of vitamin B_6 degradation. Purification and properties of 5-pyridoxic acid oxygenase from *Arthrobacter*. *J. Biol. Chem.* **261,** 15115–15120

13 Korytnyk, W. and Ikawa, M. (1970). Synthesis of vitamin B_6 analogs. *Meth. Enzymol.* **18A,** 524–566

14 Coburn, S.P. and Mahuren, J.D. (1983). A versatile cation-exchange procedure for measuring the seven major forms of vitamin B_6 in biological samples. *Anal. Biochem.* **129,** 310–317

15 Mahuren, J.D. and Coburn, S.P. (1990). B_6 vitamers:cation exchange HPLC. *J. Nutr. Biochem.* **1,** 659–663
16 Coburn, S.P. Mahuren, J.D., Wostmann, B.S., Snyder, D.L., and Townsend, D.W. (1989). Role of intestinal microflora in the metabolism of vitamin B_6 and 4′-deoxypyridoxine examined using germfree guinea pigs and rats. *J. Nutr.* **119,** 181–188
17 Collipp, P.J., Chen, S.Y., Sharma, R.K., Balanchandar, V., and Maddiah, V.T. (1975). Tryptophan metabolism in bronchial asthma. *Ann. Allergy* **35,** 153–158
18 Coleman, M. (1988). Studies of the administration of pyridoxine to children with Down's syndrome. In *Clinical and Physiological Applications of Vitamin B_6* (J.E. Leklem and R.D. Reynolds, eds.), pp. 317–328, Alan R. Liss, N.Y.
19 Barthelemy, C., Martineau, J., Bruneau, N., Muh, J.P., Lelord, G., and Callaway, E. (1988). Clinical and biological effects of pyridoxine plus magnesium in autistic subjects. In *Clinical and Physiological Applications of Vitamin B_6* (J.E. Leklem and R.D. Reynolds, eds.), pp. 329–356, Alan R. Liss, N.Y.
20 Kleijnen, J., Ter Riet, G., and Knipschild, P. (1990). Vitamin B_6 in the treatment of premenstrual syndrome—a review. *Br. J. Obstet. Gynecol.* **97,** 847–852
21 Kasdan, M.L. and Janes, C. (1987). Carpal tunnel syndrome and vitamin B_6. *Plast. Reconstr. Surg.* **79,** 456–459
22 Bendich, A. and Cohen, M. (1990). Vitamin B_6 safety issues. *Ann. NY Acad. Sci.* **585,** 321–330