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METABOLIC DISPOSITION OF PYRAZINAMIDE IN THE RAT: IDENTIFICATION OF A NOVEL *IN VIVO* METABOLITE COMMON TO BOTH RAT AND HUMAN

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ABSTRACT

Only limited studies have been reported on the disposition and pharmacokinetics of pyrazinamide (PZA) in both animals and humans. The metabolism of PZA has never been completely elucidated, consequently the metabolites of PZA, pyrazinoic acid (PA), 5-hydroxypyrazinoic acid (5-HOPA), and 5-hydroxypyrazinamide (5-HOPZA) were characterized and the disposition of PZA was examined following administration of 150 mg kg^{-1} of ^{14}C-PZA to male Wistar rats. Comparable $t_{1/2}$ for total radiolabel ^{14}C (1·45 ± 0·06 h) and PZA (1·39 ± 0·04 h) in the blood compartment were observed. Cumulative 48 h excretion in urine and faeces accounted for 82·6 ± 3·2 per cent and 11·0 ± 1·3 per cent, respectively, of the dose administered. In the 0–6 h urine collections PA, 5-HOPA, 5-HOPZA, and PZA, respectively, accounted for 25·4 ± 1·7, 17·7 ± 1·2, 11·6 ± 0·8, and 2·7 ± 0·2 per cent of the administered dose. In the 6–12 h urine samples the proportions of PA and 5-HOPA increased statistically over the 0–6 h excretion whereas 5-HOPZA decreased. Administration of PZA to humans indicated 5-HOPZA was a major urinary metabolite in human. These data suggested that direct hydroxylation of PZA was an alternative pathway in the oxidation of PZA of importance to both human and rat.

KEY WORDS Pyrazinamide Metabolism Rat Humans 5-Hydroxypyrazinamide

INTRODUCTION

Pyrazinamide (PZA), usually in combination with isoniazid, was used in the early 1950s as a first line chemotherapy regimen for tuberculosis [1–3] but, because of the high incidence of hepatotoxicity (2–18 per cent),[2,4–6] its use was abandoned. In 1970 the British Medical Research Council began to study short course (3–9 months) chemotherapy regimens for tuberculosis and reintroduced PZA into primary treatment regimens.[7] It is now established as

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an important drug in short-term chemotherapy of tuberculosis.[8–11] Several studies have shown that it speeds the attainment of culture negativity and reduces the bacteriological relapse rate after the end of chemotherapy. It has a valuable sterilizing ability in the presence of initial resistance to isoniazid or streptomycin, or both, and consequently is recognized by the WHO as one of the three major armaments against tuberculosis.[9]

The metabolism of PZA has never been completely elucidated. Weiner and Tinker[12] reported PZA, pyrazinoic acid (PA), 5-hydroxypyrazinoic acid (5-HOPA), pyrazinuric acid (PZU), and 'Compound II' as urinary excretion products in the dog. Other studies have confirmed, PA, 5-HOPA, and 'Compound II' as major urinary metabolites[13–16] but urinary recoveries were always low, ranging from 40–70 per cent of the dose.[12,14,16] Recently Pitrè *et al.*,[17] examining metabolism by rat hepatic subcellular fractions demonstrated that 5-hydroxypyrazinamide (5-HOPZA) was an *in vitro* metabolite. No attempt was made, however, to establish its presence *in vivo* or confirm it to be 'Compound II'. On the other hand, 'Compound II' has been shown to be polarographically similar to 5-HOPA[12] and chromatographically was demonstrated to be a major constituent in the urines of dogs,[12] humans,[13] guinea pigs, and Yorkshire pigs (unpublished observations). Consequently the objectives of this study were to first characterize 'Compound II' and then examine the qualitative and quantitative disposition of ^{14}C-PZA (150 mg kg^{-1} po) in male Wistar rats.

MATERIALS AND METHODS

Chemicals

Radiolabelled PZA was synthesized from pyrazine-2-[^{14}C] carboxylic acid (Amersham, Oakville, Ont.) which had a specific activity of 18·1 MBq mg^{-1}. The radiolabelled starting material was diluted with approximately 100 mg of PA (Aldrich Chemical Company, Milwaukee, WI.) and the mixture esterified by bubbling a methanolic suspension of the acid with hydrogen chloride until completely dissolved. The methyl ester was isolated, redissolved in methanol, and treated with ammonia to give ^{14}C-PZA with a specific activity of 152 μBq mg^{-1}. 5-HOPA was a generous gift from Dr I. M. Weiner (State University of New York, Syracuse, N.Y.). All other reagents were purchased from local suppliers unless otherwise indicated.

Animals

Male Wistar rats were purchased from Canadian Breeding Farms Ltd (St. Constant, P.Q.). Rats, housed in stainless steel cages with access to food and water *ad lib* were acclimatized to the animal facility for at least one week prior to use.

Treatment

PZA (150 mg kg^{-1}) was dissolved in warm water and administered at 7·5 ml kg^{-1} by gastric intubation to overnight-fasted rats weighing approximately 200 g. Radiolabelled PZA, when incorporated into the dosing solution, was administered at 0·74 MBq kg^{-1}. A fasted male Caucasian weighing 80 kg was also treated orally with 1 g of PZA dissolved in 75 ml of warm water.

Sample collections

Blood samples (10–20 µl for radiolabelled analysis and 75–100 µl for high pressure liquid chromatography (HPLC) analysis) were collected from the rat tail at various times up to 8 h. Urine collections at 0–6, 6–12, 12–24, and 24–48 h, and 0–48 h faeces were collected from similarly treated rats housed individually in stainless steel metabolic cages.

Human urine samples were collected at various intervals up to 24 h. Preliminary examination using the methods of Auscher *et al.*[13] indicated that the 3·0 to 7·5 h urine sample contained the highest concentration of 'Compound II'; consequently this urine collection was our source of human material for all further studies on the identification of this unknown.

Analysis of biological material

Blood concentrations of unlabelled PZA were determined by HPLC as previously described.[18]

Total radioactivity in blood, urine, and faeces was analysed by liquid scintillation counting (LSC). Blood (10–20 µl) and urine 25 µl were analysed as described by Thomas *et al.*[19] Faeces samples were hydrated overnight with 25 ml of water and the following day each made to 50 ml. Aliquots (1·0 ml) of each of the suspensions were placed in Combusto-cones containing Combusto-pads (Packard Instrument Canada Ltd, Mississauga, Ont.) and air-dried. Samples were then prepared for LSC using a Tissue Oxidizer (model B306 Packard Instrument Canada Ltd, Mississuaga, Ont.) and Oxisorb/Oxiprep (New England Nuclear, Boston, MA.) as described by Thomas *et al.*[19]

Thin-layer chromatography (TLC) coupled with LSC were employed to quantitatively determine PZA and its metabolites in the urines. Immediately prior to spotting samples, 1 ml aliquots of each of the urines were adjusted to pH 7·5 and then 10 µl were applied to plastic backed anion exchange TLC plates (Avicel PEI cellulose F 100 µM, 20 × 20 cm, Mandel Scientific Co., Rockwood, Ont.). Plates were developed in the same direction using an ascending dual solvent system; a neutral system of butanol:methanol:water (60:20:20) followed by an acidic system of butanol:methanol:water:acetic acid (60:20:20:1). Developed plates were serially sectioned into 5 mm strips and analysed by LSC using 15 ml of Aquasol (New England Nuclear, Boston, MA.).

Isolation of 'Compound II'

Aliquots of human urine were subjected to column chromatography as described by Auscher *et al.*[13] Peak 'P'[13] was eluted with 0·01N HCl from a 1·1 × 4 cm cationic (hydrogen form) Bio-Rad AG 50 W-X8, 200–400 mesh, packed column. PZA and its metabolites were individually separated from peak 'P' on an anionic (chloride form) Bio-Rad AG1-X8 100–200 mesh packed column (1 × 17 cm) as described.[13] 'Compound II' was collected, freeze-dried, dissolved in a small aliquot of distilled water containing 1 per cent butanol and separated by Sephadex G-10 column (0·9 × 45 cm) chromatography with 1 per cent butanol (19 ml h^{-1}). 'Compound II' eluted between 30 and 90 ml. To yield greater quantities of 'Compound II' for authentication, scaled-up versions of the above procedures were used.

Characterization of 'Compound II'

The chromatographic, spectrophotometric, and spectrometric characteristics of 'Compound II', PZA, PA, and 5-HOPA were examined. Column chromatography procedures were those of Auscher *et al.*[13] Anion exchange TLC separations were carried as described above.

The spectrophotometric characteristics of 'Compound II' in 0·01M HCl were examined by scanning between 200 and 400 nm using an HP 8450 diode array detector (Hewlett Packard Canada Ltd, Ottawa, Ont.). Authentic standards of PZA, PA, and 5-HOPA were similarly scanned for comparative purposes.

Direct probe mass spectra of the above standards and the unknown were recorded on a HP 5985 Mass Spectrometer (Hewlett Packard Canada Ltd, Ottawa, Ont.) at an ionization potential of 70 eV and a probe temperature of 25° programmed to 250° at 25° min^{-1}.

Proton nuclear magnetic resonance (PMR) spectra were recorded at ambient temperature on a Bruker WP-80 Fourier transform spectrometer (Bruker Spectrospin Canada Ltd, Milton, Ont.) at 80 MHz. Chemical shifts (δ) were measured in ppm down field from tetramethylsilane (TMS) in a solution of deuterated dimethylsulfoxide (dmso-d_6). Spectra were recorded using a flip angle of 18° (1 μs). Twelve to 1000 scans, depending on the amount of material available (0·5 to 30 mg per 0·5 ml of dmso-d_6), of 1200 Hz sweep width were stored for a 4000 data point output following Fourier transformation. A 200 MHz spectrum was also recorded for 'Compound II' on a Varian XL-200 spectrometer (Varian Canada Inc., Guelph, Ont.) for the determination of coupling constant.

Confirmation of the structure of 5-HOPZA

The structure of 'Compound II' from human urine was confirmed to be 5-HOPZA by converting it to the methoxy derivative for comparison with authentic 5-methoxy-PZA. Authentic 5-HOPA (3 mg) was dissolved in methanol (5 ml) to which was added 0·5 ml of methyl iodide and 50 mg of

sodium bicarbonate. The mixture was refluxed for 3 h. TLC (silica gel/chloroform:methanol, 1:1) showed a single spot, different from starting material, and mass spectrometry indicated that the product was 5-methoxypyrazinoic acid methyl ester (i.e. its molecular ion appeared at m/z 168). All available product from this reaction (approximately 2 mg) was dissolved in concentrated ammonia (2 ml) and was stirred for 1 h to give the 5-methoxyamide. TLC (silica gel/chloroform:methanol, 3:1) on the reaction mixture indicated that authentic 5-methoxypyrazinamide moved as a single spot different from the starting material with spectroscopic and chromatographic data congruent with its structure (see Results, Table 2, column 2). For comparison purposes 3-hydroxypyrazinamide (3-HOPZA) was synthesized by a previously described condensation reaction[20] using glyoxal and 2-aminomalonamide as starting material. The 3-methoxy derivative was prepared by treating 3-HOPZA with methyl iodide as described above. 'Compound II' (approximately 1 mg) was similarly methylated with methyl iodide for comparison with the methoxy standards.

RESULTS

Separation, characterization, and authentication of 5-HOPZA

'Compound II' was isolated from human urine as described by Auscher *et al.*[13] The chromatographic, spectrophotometric, and spectrometric characteristics are described in Table 1. For comparative purposes data for PZA, PA, and 5-HOPA have been included. The base peak, 139 of the mass spectrum of 'Compound II' corresponded to $C_5H_5N_3O_2$. The m/z of 95 and 96 represented the loss of the amide similar to the fragmental loss of the amide moiety from PZA (m/z 79 and 80). In the PMR two deuterium exchangeable protons were demonstrated in 'Compound II' with chemical shifts of the order of δ7·4 and 7·7: additional evidence supporting the presences of the amide functional group. The resonances at δ7·95 and δ7·92 appeared to be triplet clusters which were poorly resolved even at 200 MHz. The last two peaks in each of the clusters were resolved sufficiently to give coupling constants of 1·1 Hz, comparable to the 1·2 Hz coupling observed with authentic 5-HOPA. The remaining peak in each of the triplet clusters was a poorly resolved shoulder, possibly the results of long range coupling with the amide.

'Compound II' was tentatively identified as hydroxypyrazinamide; however, an unequivocal assignment of the ring position of the hydroxyl group was not possible from the data in Table 1. Methylation of 'Compound II' with methyl iodide and comparison of its spectroscopic and chromatographic properties with 3-methoxy- and 5-methoxypyrazinamide indicated that, with the exception of the molecular weights, dissimilarity was observed between the methylated derivative of 'Compound II' and 3-methoxypyrazinamide (Table 2). Congruence was observed, however,

Table 1. Chromatographic, spectrophotometric, and spectrometric characteristics of pyrazinamide and its metabolites

	Pyrazinamide (PZA)	Compound II (5-HOPZA)	5-Hydroxypyrazinoic acid (5-HOPA)	Pyrazinoic acid (PA)
Chromatography				
Elution volume (ml) from Cation exchange*	0–20	0–20	0–20	0–20
Elution volume (ml) from Anion exchange†	7–30	70–90	90–120	130–160
Elution volume (ml) from Sephadex G-10‡	ND	30–45	ND	ND
R_f on PEI cellulose Anion exchange TLC§	0·88	0·50	0·05	0·33
UV spectral scan‖	λmax 269 277(s) 311	λmax 256 311	λmax 258 307	λmax 268 308
Mass spectra¶	44 (1) 53 (11) 79 (13) 80 (67) 105 (4) 107 (1) 123 (100)	44 (4) 68 (17) 95 (36) 96 (22) 111 (5) 123 (11) 139 (100)	44 (12) 53 (17) 68 (74) 95 (38) 96 (100) 123 (9) 140 (45)	45 (8) 53 (55) 70 (6) 80 (100) 106 (4) 124 (16)
PMR (dmso-d_6)**	δ9·23(d,J=1·5, 1H) δ8·89(d,J=2·6, 1H) δ8·75(q,J=1·5 and 2·4, 1H) δ8·29(sb, D_2O, 1H) δ7·92(sb, D_2O, 1H)	δ7·95(t,J=1·1, 1H) δ7·92(t,J=1·1, 1H) δ7·73(sb, D_2O, 1H) δ7·45(sb, D_2O, 1H)	δ8·07(d,J=1·2, 1H) δ8·00(d,J=1·2, 1H)	δ9·21(d,J<1·0, 1H) δ8·85(m,J=5·0 and 2·5, 2H)

* Column 1·1 × 4 cm packed with Bio-Rad AG1 50W-X8, H+, 200–400 mesh, and eluted with 0·01M HCl at a rate of ~ 40 ml h^{-1}.[13]
† Column 1·0 × 17 cm packed with Bio-Rad AG1-X8, Cl^-, 100–200 mesh was eluted at a rate of 84 ml h^{-1} with distilled water (~ 20 min) followed by linear gradient of distilled H_2O-HCl 0·05M.
‡ Column 0·9 × 45 cm packed with Sephadex G-10 and eluted with 1 per cent butanol at 19 ml h^{-1}.
§ Avicel PEI cellulose F 100 μm developed with butanol:methanol:water (60:20:20) followed by butanol:methanol:water: acetic acid (60:20:20:1).
‖ Samples dissolved in 0·01M HCl scanned 200–400 nm – (s)shoulder.
¶ Mass spectra by direct probe insertion at 70 eV. Data presented is m/z with relative abundance in parentheses.
** Chemical shifts (δ) were measured in ppm down field from TMS and multiplets indicated as (d) doublet, (t) triplet or (q) quartet, (m) multiplet, (sb) singlet broad, (D_2O) peak disappears after exchange with D_2O.

Table 2. Spectral and chromatographic properties of 3-methoxypyrazinamide, 5-methoxypyrazinamide, and methylated 'Compound II'

	3-Methoxypyrazinamide	5-Methoxypyrazinamide	Methylated 'Compound II'
PMR (dmso-d_6) ppm			
methoxy	3·69	3·34	3·33
aromatic	7·64 (d, J=4)	7·95 (d, J=0·9)	7·95 (d, J=0·9)
aromatic	8·02 (d, J=4)	8·36 (s, broad)	8·37 (s, broad)
GC-MS (m/z) relative abundance			
153 (parent ion)	98	100	100
136	–	17	8
124	–	16	13
109	70	53	51
98	–	14	8
IR (four most prominent peaks in order of intensity) wave number in cm^{-1}			
	1650	3437	3437
	1195	1662	1660
	3362	1687	1687
	1540	1595	1596
HPLC (RP-18/acetonitrile:water, 40:60) retention volume in ml			
	3·15	2·30	2·30
GLC (capillary column, DB-1, 15 metres) retention time in min			
	4·3	5·4	5·4

between methylated 'Compound II' and standard 5-methoxypyrazinamide establishing 'Compound II' as 5-HOPZA.

To establish that 5-HOPZA was in fact a urinary metabolite in the rat, urine from a ^{14}C-PZA-treated animal was separated by ion exchange column chromatography as described above. Radiolabelled 'Compound II' (5-HOPZA) was collected and rechromatographed on anion exchange cellulose TLC plates as described under 'material and methods'. Radioactivity moved as a single spot with an R_f of 0·5 relative to the solvent front. Consequently, the metabolite profiles in the urines of ^{14}C-PZA-treated rats were determined using this dual solvent TLC system.

Disposition of PZA in the male Wistar rat

Total radioactivity in the blood compartment peaked at approximately 1 h following oral administration of 150 mg kg^{-1} of ^{14}C-PZA (Figure 1) and blood concentrations of total radioactivity at 1, 2, 3, and 4 h, expressed as

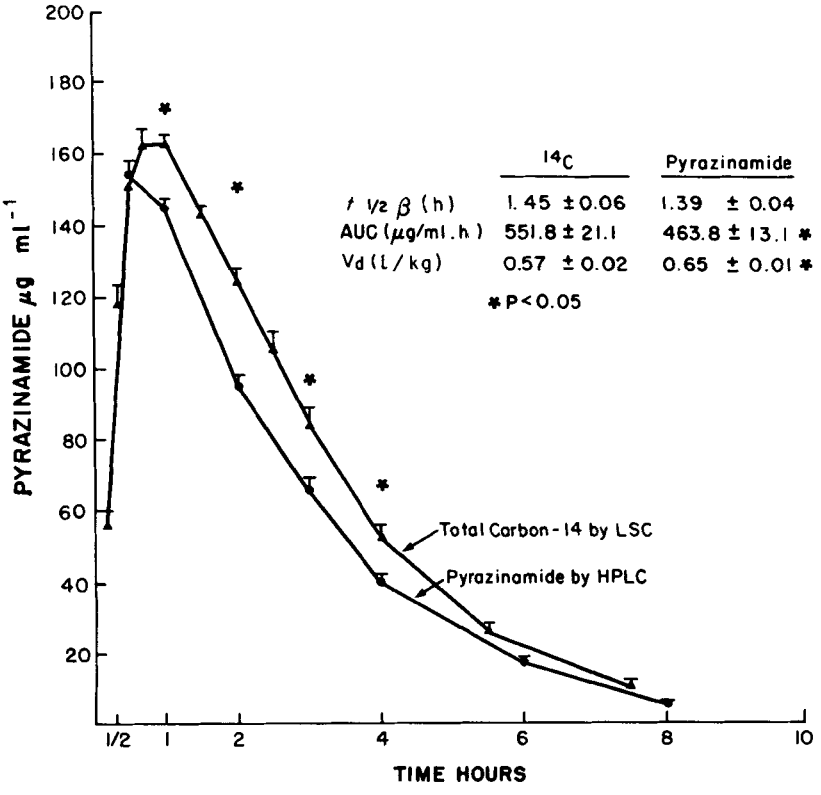

Figure 1. Blood profile of total radioactivity (▲) and pyrazinamide (●) following oral administration of 150 mg mg^{-1} of ^{14}C-pyrazinamide or unlabelled pyrazinamide. Values are means ± S.E. from five rats

Table 3. Urinary excretion profile of pyrazinamide and its metabolites in Male Wistar Rats (n = 6)

	R_f‡	0–6 h	6–12 h
Total excretion*		57·4±3·2	19·8±1·6
5-hydroxypyrazinoic acid (5-HOPA)	0·05	17·7±1·2 (30·8±1·0)	7·2±0·7 (35·9±0·7)
Pyrazinoic acid (PA)	0·33	25·4±1·7 (44·3±1·7)	9·7±0·8 (49·1±0·5)
5-hydroxypyrazinamide (5-HOPZA)	0·50	11·6±0·8 (20·2±0·7)	2·7±0·2 (13·9±0·7)
Pyrazinamide (PZA)	0·88	2·7±0·2 (4·7±0·1)	0·2±0·003 (1·2±0·04)

* Results are expressed as mean ± SE of the percentage of the dose administered. Values in parenthesis are proportions of the total in the urine.
† Using TLC method described in Analysis of Biological Material section.

pyrazinamide, were significantly higher than the actual concentration of unchanged PZA in the plasma as determined by HPLC. Unchanged PZA, however, accounted for 70 per cent of the radioactivity in the blood compartment during the first 4 h. Both the carbon-14 and the HPLC (pyrazinamide) data gave comparable $t_{½}\beta$ for elimination from the blood compartment (Figure 1, inset). The AUC was, however, 20 per cent higher and the V_d 12 per cent lower when computations were made using the ^{14}C data.

Cumulative urinary excretion of total radioactivity at 6, 12, 24, and 48 h was 57·4 ± 3·2, 77·2 ± 3·2, 81·7 ± 3·2, and 82·6 ± 3·2, respectively, while 11·0 ± 1·3 per cent of the administered dose was recovered in the faeces at 48 h.

Urinary metabolite profiles of the 0–6 and 6–12 h urines indicated that, in the male Wistar rat, PA was the major metabolite (Table 3). The prevalence of the other compounds in the urine in decreasing order were 5-HOPA, 5-HOPZA, and PZA. The ratios of each of the metabolites to total radioactivity excreted, tended to increase in the 6–12 h urine samples, with the exception of 5-HOPZA which appeared to be higher in the early (0–6 h) urine samples.

DISCUSSION

'Compound II' first reported by Weiner and Tinker,[12] alluded to by Ellard and Haslam[21] and finally isolated by Auscher *et al.*[13] was characterized as 5-HOPZA using various chromatographic, spectrometric, and synthetic techniques (Tables 1 and 2). To our knowledge this is the first report

establishing 5-HOPZA as an *in vivo* urinary metabolite of PZA common to both the human and rat. Pitrè *et al.*[17] indicated 5-HOPZA was a product of the *in vitro* incubation of PZA with rat liver preparations; however, no attempt was made to establish its presence *in vivo*.

A comparison of the blood profile and kinetic parameters for ^{14}C and unchanged PZA (Figure 1) indicated that PZA and its metabolites did not accumulate in the blood compartment of the rat; metabolites, once formed, were readily eliminated from the blood compartment resulting in the half-life for PZA of approximately 1·4 h. Ellard[15] indicated the half-life of PZA in the blood compartment of humans was 9–10 h. More recent data[14,18] have confirmed these findings indicating that humans have a five-fold longer pyrazinamide half-life than the rat.

In contrast to previous studies[12,14,16] in which only 40–70 per cent recovery of the administered dose of PZA has been observed, we were able to recover approximately 93 per cent of the dose of radiolabelled PZA in the faeces and urine. The higher recovery can be attributed to two facts. Biliary excretion, neglected in previous studies, was accounted for in this study by examination of the faeces. Incomplete recovery or lack of recovery of one or more of the metabolites in the sample work-up procedures of previous studies were real possibilities which we circumvented by using radiolabelled PZA.

The metabolite profile in 0–6 h and 6–12 h urines (Table 3) indicated that PZA was extensively metabolized by the rat (< 3 per cent of the dose excreted unchanged in the 0–12 h collection). 5-HOPZA was a significant urinary metabolite, accounting in the first 12 h for approximately 14·3 per cent of the dose. In contrast to 5-HOPA and PA, the ratio of 5-HOPZA excretion to total radioactivity was higher for the 0–6 h urine collection than for the 6–12 h collection (Table 3), suggesting that 5-HOPZA was preferentially eliminated in the early phase of the elimination profile when PZA concentrations were high. A comparison of the data for human[13] with the cumulative 0–12 h rat data (Table 3) indicated that rat and human excreted identical metabolites with PA being excreted by both in approximately the same proportion to total excretion (human 0·42 vs rat 0·45). Rat, however, appeared to preferentially eliminate 5-HOPA (rat 0·32 vs human 0·22) whereas human showed a greater propensity for the excretion of 5-HOPZA (human 0·28 vs rat 0·19).

The enzyme responsible for the 5-hydroxylation of PZA has not been adequately characterized. Xanthine oxidase, the enzyme responsible for the hydroxylation of PA[12] does not appear to be responsible for the 5-hydroxylation of PZA in humans, since it was observed that a xanthinuric patient continued to excrete a significant amount of 5-HOPZA in the urine but only traces of 5-HOPA.[13] On the other hand, Pitrè *et al.*,[17] on the basis of subcellular fractionation studies and xanthine oxidase inhibitor (allopurinol 10^{-3} m) studies, concluded that xanthine oxidase was responsible for the *in vitro* 5-hydroxylation of PZA in the rat.

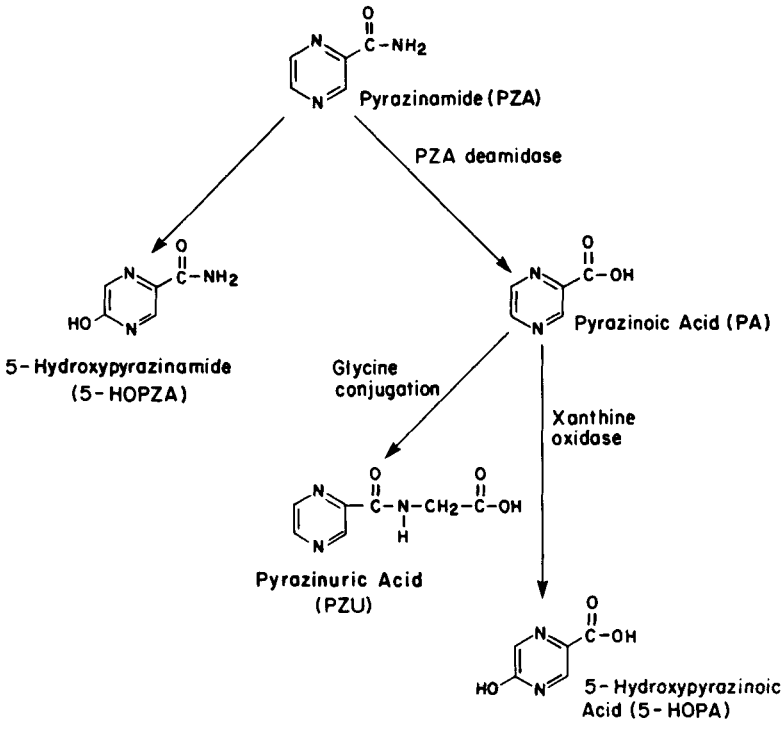

Figure 2. Summary of currently accepted metabolic pathways of pyrazinamide disposition in mammalians

Our results demonstrate that in the rat 5-HOPZA is a major *in vivo* metabolite of PZA. Its early appearance in the urine suggests that it is the product of the direct oxidative metabolism of PZA. The fact that a compound with chromatographic characteristics similar to 5-HOPZA has been observed also in the urine of dogs,[12] humans,[12,13,15] guinea pigs, and Yorkshire pigs (unpublished observations) suggests that this oxidative pathway is common to both animals and humans and represents another important alternative oxidative pathway in the overall metabolic disposition of PZA by mammalians (Figure 2).

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