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Note

Identification of 5-hydroxypyrazinamide isolated from urine of subjects given pyrazinamide

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Since the early 1950s, pyrazinamide (PZA) has been known to have specific activity against *Mycobacterium tuberculosis* [1–3]. Although PZA proved to be effective for the treatment of chronic tuberculosis, especially when resistance phenomena to the classic antituberculosis agents arose, its utility was questioned because of adverse reactions and the uncertainty of what the therapeutic dose should be [4].

During the last fifteen years, advanced criteria in the primary chemotherapy of tuberculosis have shown PZA to be a valuable antituberculosis agent when combined with other drugs (e.g., rifampicin, isoniazid, streptomycin) in the so-called short-course treatment of the disease [4].

The pharmacokinetic aspects of PZA treatment of humans were studied by Caccia [5], Grassi et al. [6], Stottmeier et al. [7], Ellard [8], Boulahbal et al. [9] and Roboz et al. [10], using different methods for the assessment of PZA and/or its metabolites in biological fluids. Moreover, PZA metabolites such as pyrazinoic acid (PA) [8, 10–12], 5-hydroxypyrazinoic acid (5-OH-PA) [10, 12] and, in minor amounts, pyrazinuric acid (PUA) [12] were found in the plasma and/or urine of humans and other animal species. Weiner and Tinker [12] detected another PZA transformation product in the urine of men and dogs but did not identify it.

In 1981, Pitrè et al. [13] identified 5-hydroxypyrazinamide (5-OH-PZA) in rat liver microsome preparations incubated with pyrazinamide.

This paper describes the isolation of a PZA transformation product (I), not previously identified in human urine, and its identification as 5-OH-PZA by physicochemical methods.

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EXPERIMENTAL

Chemicals

Chloroform, methanol, ethyl acetate and standard pyrazinamide and pyrazinoic acid were of analytical-reagent grade. 5-Hydroxypyrazinamide and 5-hydroxypyrazinoic acid were supplied by D. Pitrè (Bracco, Milan, Italy) and 6-hydroxypyrazinamide by H. Foks (University of Gdansk, Gdansk, Poland) [14]. Nitrogen was of gas chromatography quality.

Materials and glassware

Screw-capped glass centrifuge tubes and conical glass tubes were washed in a Miele machine with final rinsing with deionized water. Silica gel 60 F_{254} precoated plates were obtained from Merck (Darmstadt, F.R.G.) and a Camag lamp (Bracco) with UV light at 254 nm was used to view them. A Continental Alter 2864 horizontal shaker (Passoni, Milan, Italy) was used for the elution of the silica gel powders. Bransonic 12 ultrasonic equipment (Smithkline, Soest, The Netherlands) and vortex agitators were used for thoroughly mixing solids in the organic solvent and for improving the solubilization of dried residues. Stainless-steel lancets were employed for scraping the silica gel plates.

The 24-h urine collections from humans who took PZA* were stored at −22°C until lyophilized.

Procedure for isolation of I

Preliminary thin-layer chromatography (TLC) assays of the lyophilized urine from volunteers indicated the presence of I, a compound more polar than PZA and less polar than PA. Because of the poor extraction of PZA into organic solvents immiscible with water and the greater polarity of I than of PZA, a means of isolating I from urine other than solvent extraction was developed, starting from lyophilized urine.

Urine residues (4 g from 70–80 ml of urine) from both an untreated (B) and a treated (T) subject were vortexed with 36 ml of methanol in screw-capped glass tubes, as TLC showed that I was extracted into methanol in the above proportion in a preliminary separation microassay.

To the centrifuged methanol extract diluted 1:1 with methanol, ten volumes of ethyl acetate were added dropwise. Together with some urinary components, mostly PA and 5-OH-PA were precipitated, while most of I remained in solution, as ascertained by TLC assays of solution samples after adding ethyl acetate (Fig. 1). After centrifuging, the supernatants from B and T were evaporated to dryness in conical glass tubes under a stream of nitrogen in a water-bath at 37°C. From the residues vortexed with 3–4 ml of ethyl acetate–methanol (7:3) and then centrifuged, most of I was recovered in the T supernatant.

The ethyl acetate–methanol (7:3) supernatants from the B and T tubes were

*PZA (Piraldina, 500-mg tablets, supplied by Bracco) was given orally (15 mg/kg) to healthy volunteers in a clinical trial conducted by Dr. G. Buniva of the Clinical Research Department of Gruppo Lepetit.

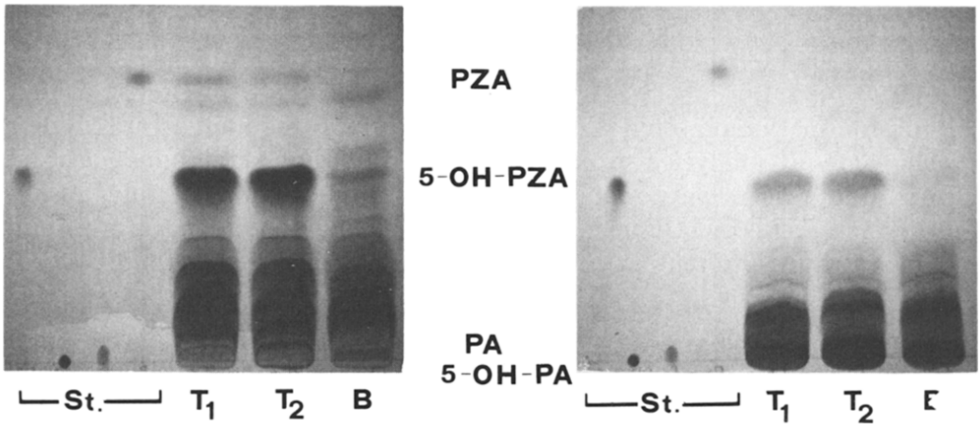


Fig. 1. TLC of supernatants (left) and precipitates (right) in the ethyl acetate–methanol (10:1) mixtures from B (one subject) and T (two subjects) urine after development in chloroform–methanol (85:15) for 2 h and then in chloroform–methanol (7:3) for 2.5 h. St. = reference standards.

applied to chromatographic plates as 14-cm bands. The plates were initially chromatographed in chloroform–methanol (85:15) for 2 h and then in chloroform–methanol (7:3) for an additional 2.5 h. Standard PZA and 5-OH-PZA served as references for the location of I. The large bands corresponding to I, which had the same R_F as 5-OH-PZA, were scraped off from every T plate and transferred to centrifuge tubes. Zones of the same size as I zones and at the same distance from the starting line were analogously scraped off from the B plates.

Methanol (7.0 ml) was added to each tube, which was sonicated for 5 min, agitated on a horizontal shaker and centrifuged. The supernatants were placed in conical glass tubes, to which the centrifuged methanol washings of the wet silica gel powder were also added. After drying under a stream of nitrogen, the residues were taken up four times with methanol (0.5 ml at a time) in order to recover most of the compound to be isolated.

For further purification, these methanol solutions from the selected bands of the B and T plates were again chromatographed for 2.5 h in chloroform–methanol (7:3) and subsequently processed, as previously described, to obtain the residues which were submitted to spectroscopic characterization.

Several milligrams of purified urinary metabolite of PZA (compound I) were obtained from sample T by the isolation procedure described, which involved only simple separation steps and common laboratory instruments. This amount was enough to elucidate its structure by spectroscopic techniques without any derivatization using sample B as a blank.

Instrumentation

Electron-impact mass spectra were obtained with a Hewlett-Packard 5985B mass spectrometer at 70 eV with a direct-inlet system heated at 15 °C/min from 70 to 200 °C; the ion-source temperature was 200 °C.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained at 270

TABLE I

SELECTED ELECTRON-IMPACT MASS SPECTRAL DATA FOR PYRAZINAMIDE (PZA), COMPOUND I, 5-HYDROXYPYRAZINAMIDE (5-OH-PZA) AND 6-HYDROXYPYRAZINAMIDE (6-OH-PZA)

Compound	Structural assignments*					
	$[M]^{+\cdot}$	$[M-NH_2]^+$	$[M-CO]^{+\cdot}$	$[M-HNCO]^{+\cdot}$	$[M-CONH_2]^+$	$[M-HNCO-CO]^{+\cdot}$
PZA	123 (74)	107 (1)	95 (1)	80 (100)	79 (22)	–
I	139 (100)	123 (13)	111 (8)	96 (31)	95 (45)	68 (58)
5-OH-PZA	139 (100)	123 (12)	111 (8)	96 (30)	95 (58)	68 (65)
6-OH-PZA	139 (100)	123 (2)	111 (5)	96 (74)	95 (30)	68 (70)

*Results shown are *m/z*, with relative intensity in parentheses.

MHz in C^2HCl_3 solution, with tetramethylsilane (TMS) as the internal reference, on a Bruker WH-270 FT NMR cryo-spectrometer equipped with a 36 K BNC-12 computer and disk unit.

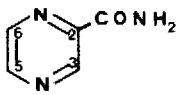
RESULTS

The most characteristic ions in the mass spectrum of I are listed in Table I. The molecular ion at *m/z* 139, 16 mass units more than PZA, indicates the presence of an oxygen derived from a hydroxy group attached directly to the pyrazine ring, as demonstrated by the ion at *m/z* 111 $[M-CO]^{+\cdot}$, which is typical of phenols. The location of the OH group in position 3 on the pyrazine ring can be excluded because the loss of NH_3 due to an *ortho* effect was not observed [13]. The study of the fragmentation pattern of I cannot itself determine which of the two remaining positions (5 or 6) is more likely; in fact, the differences between the electron-impact mass spectra of *meta* and *para* isomers previously reported in the literature are usually small [15].

TABLE II

ASSIGNMENTS OF THE SIGNALS OF THE ^1H NMR SPECTRA OF COMPOUND I AND 5-HYDROXYPYRAZINAMIDE (5-OH-PZA)

The attributions were based on selective decoupling experiments and addition of ^2H_2O to the DMSO-d_6 solution.



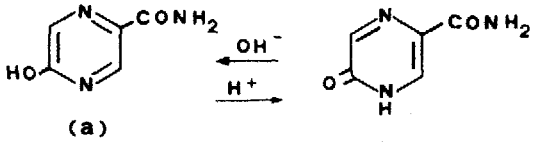
PZA

Compound	Solvent	δ (ppm)	
		NH_2	H-3 and H-6
I	DMSO-d_6	7.35; 6.87	8.16; 7.38
5-OH-PZA	DMSO-d_6	7.72; 7.47	7.96*
5-OH-PZA and I	DMSO-d_6 + trace of ^2HCl	–	7.96*

*Two protons.

For this reason it appeared useful to examine the standard compounds 5-OH-PZA and 6-OH-PZA, which were analysed under the same experimental conditions as I (Table I). As expected, all the relevant ions of the two isomers had the same *m/z* values, but their relative intensities showed significant differences which indicate that I is 5-OH-PZA. This result was confirmed by ^{1}H NMR spectroscopy, a technique already applied successfully for characterizing positional isomers of pyrazinamide derivatives [16, 17].

The spectra of I and 5-OH-PZA obtained in DMSO-d_6 solution are different, but they become the same after addition of a trace of ^{2}HCl to DMSO-d_6 (Table II). The change in the ^{1}H NMR spectrum of 5-OH-PZA after addition of ^{2}HCl to the DMSO-d_6 solution is interpreted as being due to the presence of 5-OH-PZA in two tautomeric forms, a and b shown below. In fact, the hydroxy form is expected to exist in alkaline medium and the keto form in neutral or acidic medium, according to the following equilibrium [18]:



(a) (b)

The identity of the spectra of I and 5-OH-PZA obtained under the same pH conditions demonstrated that the two compounds have the same structure.

DISCUSSION

The findings of this investigation provide more information about the in vivo metabolism of PZA. In addition to the already known PA, 5-OH-PA and PUA, 5-OH-PZA is now recognized as another product of the transformation of PZA by the human body.

The presence of 5-OH-PZA in human urine is evidence of direct oxidation of the PZA molecule, the first biotransformation product of which was long believed to be PA. In fact, as no PZA metabolite with only one hydroxy group in the pyrazine ring had ever been identified in previous in vivo studies of the PZA metabolism, 5-OH-PA in biological fluids of PZA-treated animals (man included) had been thought to result from oxidation of PA, previously formed from PZA by enzymatic hydrolysis with an amidase. The in vitro formation of 5-OH-PZA from PZA by rat liver microsomes allowed Pitrè et al. [13] to indicate a first alternative metabolic step for the parent drug (PZA→5-OH-PZA) in addition to one already known (PZA→PA) and, consequently, 5-OH-PA might also be formed by biological cleavage of the amide group of 5-OH-PZA.

Our investigation provides the experimental evidence for the in vivo formation of 5-OH-PZA. Probably the unidentified compound "II" detected by Weiner and Tinker [12] in the urines of a dog and a man was 5-OH-PZA. In fact, this compound was said to have "the same polarographic characteristics as 5-OH-PA, but different electrophoretic mobility" and "was never detected after pyrazinoate

administration", which is in accord with the structure of 5-OH-PZA.

As the study described here had only the aim of isolating and identifying a PZA metabolite different from those already recognized in human urine, no additional investigation of its quantitative importance in the recovery of administered PZA was carried out.

After assessment of PZA, PA and 5-OH-PA in the urine of a healthy subject, Ellard and Haslam [19] found that the urinary recovery of PZA was incomplete (about 75% of the dose). As the other known urinary metabolite PUA would represent only a very modest amount of the administered PZA according to Weiner and Tinker [12], the urinary recovery of the drug can be increased with the additional determination of 5-OH-PZA, which is now identified, but has been shown to be quantitatively less important than PA and 5-OH-PA [12, 19].

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