Detection and Identification of Some Metabolites of *iso*Nicotinic Acid Hydrazide (Isoniazid) in Human Urine

By W. F. J. CUTHBERTSON, D. M. IRELAND AND W. WOLFF

Glaxo Laboratories Ltd., Greenford, Middlesex

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Kelly, Poet & Chesner (1952) have investigated the metabolic fate of isoniazid (pyridine-4-carboxyhydrazide) in dogs. After the oral administration of isoniazid (5 and 20 mg./kg. body weight), 20–50 % of the drug was excreted unchanged in the first and 1–5 % in the second 24 hr. *iso*Nicotinic acid was isolated and identified as a major metabolite, 20–40 % of the dose being excreted in this form. They also reported the possible presence of small amounts of *N'*-methylisonicotinic acid hydrazide in the urine, but they could not identify *N'*-methylisonicotinamide or isonicotinamide as metabolites.

We have studied qualitatively by means of paper chromatography and ionophoresis the tertiary pyridine metabolites in urines of human subjects dosed with isoniazid. Our results suggest some differences between the metabolism of isoniazid in men and that in dogs. A preliminary communication of the methods used for detecting and identifying tertiary pyridine compounds has been published (Cuthbertson & Ireland, 1952).

EXPERIMENTAL AND RESULTS

Human urine. Six 24-hr. urines were collected from patients undergoing isoniazid therapy who received 100 mg. isoniazid orally every 12 hr., irrespective of their body weight: all these patients had been treated with isoniazid at

this level for at least 2 months. Five of the subjects were under treatment with streptomycin (1 g. 3 or 7 times weekly) and isoniazid, while one was receiving *p*-aminosalicylic acid in addition to streptomycin and isoniazid. Neither streptomycin nor *p*-aminosalicylic acid interfered with the detection of the tertiary pyridine compounds on paper chromatograms.

Healthy subjects not undergoing isoniazid therapy provided 24 hr. urines for controls.

Reference compounds. The compounds listed in Table 1 were obtained commercially or as gifts, except isonicotinoylglycine, which was prepared by the method of Fox & Field (1943) for nicotinoylglycine.

Paper chromatography. This was carried out by the descending technique with Whatman no. 1 and Whatman no. 4 papers at room temperature. The solvent front was allowed to travel about 36 cm. Slight variations in R_F values, particularly in urine samples, were prevented by running reference compounds on each chromatogram. For urines or urine concentrates, on account of the concentration of solids, the reference compounds were added to a sample prepared from control urine. The solvent systems used were *n*-butanol saturated with water and ethyl methyl ketone:acetic acid: water (49:1:50; Kodicek & Reddi, 1951) and *n*-propanol: water (4:1). After development the paper was air-dried. The R_F values (Whatman no. 1 paper) for nicotinoylglycine in *n*-butanol:water and *n*-propanol:water were 0.08 and 0.35 respectively; the corresponding values for isonicotinoylglycine were 0.08 and 0.33. The R_F values for the other compounds listed in Table 1 have been recorded

Table 1. Colours produced on paper by some tertiary pyridine compounds in the König reaction

(Colours obtained from 10 µg. of compound on Whatman no. 4 paper. Colour of fluorescence in ultraviolet light is given in parentheses.)

Compound	Colour after cyanogen bromide treatment	Colour with condensing reagents applied after cyanogen bromide treatment			
		<i>p</i> -Aminobenzoic acid	Benzidine	3-Methyl-1-phenyl-pyrazolone	<i>p</i> -Aminoacetophenone
Nicotinic acid	Pale yellow	Yellow (Yellow)	Red	Red-yellow turning brown-green	Orange
<i>iso</i> Nicotinic acid	None	None	Pale light blue	Pale purple turning blue	Pale pink
Nicotinamide	Pale yellow	Yellow (Yellow)	Orange-red	Red turning green	Yellow
<i>iso</i> Nicotinamide	None	None	Purple	Purple turning blue	Pink
Nicotinic acid hydrazide	Pale yellow	Yellow (Yellow)	Orange	Yellow-orange	Yellow
Isoniazid	None (Light-blue)	Very pale yellow (Light-blue)	Pale yellow-green	Pale green	Pale yellow
Nicotinoylglycine	Pale yellow	Pale pink	—	Red-brown turning pink	—
<i>iso</i> Nicotinoylglycine	Purple	Fugitive purple	Blue	Purple turning blue	—

(Cuthbertson & Ireland, 1952). The R_F values of the nicotinic acid derivatives are almost identical with those of the corresponding isonicotinic acid derivatives. Within each series the R_F values of the acids, amides and hydrazides are well separated. The R_F values of the hydrazides and amides are not greatly affected by the pH of the aqueous phase of the solvent system, whereas the R_F values of the acidic compounds alter with the pH of the solvent, as found by Kodicek & Reddi (1951).

Paper ionophoresis. The apparatus of Latner (1952) and Whatman no. 4 and no. 1 papers were used with 0.05 M-phosphate buffer, pH 6.5, and an applied potential of 250 v for 4 hr. or 0.2 M-potassium hydrogen phthalate buffer, pH 4.0, and an applied potential of 120 v for 15 hr. After the run the paper was air dried. In phosphate buffer, pH 6.5, the acids moved towards the anode, whereas the basic compounds remained on or near the point of application. The mobilities of nicotinic acid and isonicotinic acid were identical but greater than the mobilities of nicotinoyl and isonicotinoylglycine, which also were identical. In phthalate buffer, pH 4.0, the basic compounds moved towards the cathode, whereas the acids remained on or near the point of application. The mobilities of the amides and hydrazides were identical.

Table 2. Colours produced on paper by reaction of some tertiary pyridine compounds with picryl chloride

(Colours obtained from 10 μ g. of compound on Whatman no. 4 paper.)

Compound	Colour in	
	Air	NH ₃ vapour
Nicotinic acid	Pale yellow	Red
isoNicotinic acid	Pale yellow	Red
Nicotinamide	Pale yellow	Red
isoNicotinamide	Pale yellow	Red
Nicotinic acid hydrazide	Red	Red
Isoniazid	Purple	Purple
Nicotinoylglycine	Yellow-brown	Yellow-brown
isoNicotinoylglycine	Green	Green

Detecting reagents

Cyanogen bromide. The detection and identification of some tertiary pyridine compounds on paper chromatograms has been described by Kodicek & Reddi (1951) and Huebner (1951). The general method, based on König's (1904) reaction, was to apply to the developed and dried chromatogram cyanogen bromide followed by a primary aromatic amine. The amines used were *p*-aminobenzoic acid (Kodicek & Reddi, 1951) and benzidine (Huebner, 1951). We found that *p*-aminobenzoic acid gave excellent results with the nicotinic acid derivatives but little or no colour with 10 μ g. of the isonicotinic acid derivatives. Benzidine gave good and different colours with all the compounds, although some colours varied in reproducibility.

Cyanogen bromide was conveniently applied by leaving the dried paper over a few crystals of the reagent for 30 min. in a closed vessel. We also used as coupling reagents *p*-aminoacetophenone (1%, w/v, solution in ethanol: water, 1:1, v/v) and 3-methyl-1-phenylpyrazol-5-one (saturated aqueous solution kept in the refrigerator in a brown bottle). The latter was prepared by the method of Knorr (1883) and has been used by Epstein (1947) and Boxer & Rickards

(1951) in cyanide and cyanocobalamin assay methods respectively. The *p*-aminoacetophenone reagent was not sensitive with less than 10 μ g. of the compounds and gave fading colours. The 3-methyl-1-phenylpyrazol-5-one reagent gave characteristic colours with amounts of the compounds tested greater than 5 μ g. The colours obtained with eight tertiary pyridine compounds on paper after cyanogen bromide treatment and subsequent spraying with the various condensing reagents are summarized in Table 1. Some of the spots were fluorescent when exposed to ultraviolet light from a low pressure mercury lamp with a filter transmitting mainly at 254 m μ .

Picryl chloride. Picryl chloride (2-chloro-1:3:5-trinitrobenzene) has been used for the detection and estimation of hydrazine (Kulberg & Cherkosov, 1951). We found that this compound (1.5%, w/v, in ethanol), applied to the air-dried chromatogram by dipping the paper quickly in the reagent, gave with the tertiary pyridine compounds characteristic coloured spots after 30–60 min. exposure to air or to NH₃ vapour. About 5 μ g. could be detected with this reagent. The colours produced are summarized in Table 2. The reagent gave a useful complementary and confirmatory test for the detection of the metabolites of isoniazid.

Other reagents. 1-Fluoro-2:4-dinitrobenzene (1%, v/v, in ethanol) gave colours similar to those obtained with picryl chloride, but they developed more slowly. 1-Chloro-2:4-dinitrobenzene (1%, w/v, in ethanol), used for the estimation of isoniazid by Scott (1952), gave yellow-orange colours with the hydrazides, but was not as sensitive as picryl chloride. The reagent did not appear to react effectively with the other tertiary pyridine compounds. The hydrazides, but not the other compounds, could also be detected by spraying the dried chromatogram with dimethylaminobenzaldehyde (Dalgiesh, 1952), when orange-yellow colours developed after 24 hr. at room temperature, or by spraying with diazotized *p*-nitraniline followed by 20% Na₂CO₃ (Bray, Thorpe & White, 1950), when yellow colours immediately appeared, though these faded in a few hours.

Investigation of human urine

Samples (5 ml.) of the 24 hr. urines (pH 6–7) from patients who had ingested isoniazid were freeze-dried. The dried residue was suspended in 0.5 ml. of distilled water, and 50 μ l. of the supernatant liquid were applied to the chromatogram paper in five successive 10 μ l. portions, each spot being dried before the next 10 μ l. was applied. Concentrates prepared in the same way from urine of subjects used as controls were applied to the same paper. To similar concentrates pure isonicotinic acid derivatives (10 μ g./50 μ l.) were added. Another concentrate of control urine was prepared with 100 μ g./50 μ l. of added isoniazid. These concentrates were also applied to the paper. The chromatogram was developed with *n*-butanol: water or *n*-propanol: water and subsequently treated with cyanogen bromide and methylphenylpyrazolone or benzidine, or with picryl chloride. Examination of the treated papers showed, for urines of all six subjects, two spots not present in control urines. They had the same R_F values and colours as those obtained from the controls with added isonicotinoylglycine and isonicotinic acid respectively.

Isoniazid was detected in the concentrates of the urine of only one of the six subjects. The isoniazid in the control concentrates to which 10 μ g. had been added was easily detected. In the control concentrate with 100 μ g. isoniazid

no breakdown products of isoniazid, such as isonicotinic acid, were detected.

Hydrolysis of isonicotinoylglycine. To demonstrate the production of isonicotinic acid and glycine by hydrolysis of the metabolite thought to be isonicotinoylglycine, it was necessary to purify the urine. Urine (200 ml.) was adjusted to pH 2.5 with HCl and extracted continuously with ether for 16 hr. The residual aqueous solution was neutralized with NaOH, concentrated *in vacuo* and filtered from the precipitated solids. The final volume was 10 ml. One ml. of this solution was applied to paper as a thin streak and chromatographed with *n*-propanol:water. The band containing the isonicotinoylglycine was cut out and eluted with three successive 20 ml. of distilled water. The combined water extracts were concentrated *in vacuo* to 1 ml. which was applied to paper as a streak and subjected to ionophoresis in phosphate buffer pH 6.5. The band containing the isonicotinoylglycine was again cut out, eluted, concentrated, chromatographed and eluted as before. Part of the final eluate was chromatographed and the remainder was hydrolysed by heating at 100° for 3 hr. in 5*N*-HCl in a sealed tube. The hydrolysate was neutralized with NaOH and chromatographed. In the hydrolysed eluate isonicotinoylglycine was no longer detectable, but substances giving the same *R_F* values and colour reactions as isonicotinic acid and glycine were present. Glycine was detected by spraying with ninhydrin (0.5%, w/v, in *n*-butanol).

DISCUSSION

Elmendorf, Cawthon, Muschenheim & McDermott (1952) have reported that the 24 hr. urinary ex-

cretion of isoniazid after ingestion of a single dose (3 mg./kg.) by six human subjects varied from 47.8 to 70.7% of the dose. In five of the six cases reported here no isoniazid was detected on paper chromatograms, indicating that less than 20% of the total ingested dose of isoniazid had been excreted unchanged in 24 hr. In the one case where isoniazid excretion was detected it was calculated that about 20% of the dose had been excreted unchanged in 24 hr.

SUMMARY

1. Methods are described for detecting and identifying some tertiary pyridine compounds, in particular pyridine-4-carboxy derivatives.

2. The probable identities of two metabolites excreted in urine by human subjects treated with isoniazid (pyridine-4-carboxyhydrazide) have been established as isonicotinoylglycine and isonicotinic acid. Large amounts of unchanged isoniazid were not excreted.

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The Action of Some Water-soluble Poly- α -amino Acids on Bacteria

By E. KATCHALSKI, L. BICHOWSKI-SLOMNITZKI AND B. E. VOLCANI

The Weizmann Institute of Science, Rehovoth, Israel

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It has been shown that different bacteria and fungi secrete antibacterial polypeptides (for review, see Synge, 1949). The work of Bloom, Watson, Cromartie & Freed (1947) and Bloom & Blake (1948) showed that cells of higher animals, as well, may excrete antibacterial substances of a peptide nature. These facts encouraged us to study the biological properties of water soluble poly- α -amino acids (for review see Katchalski, 1951).

Poly-DL-alanine (Astbury, Dalglish, Darmon & Sutherland, 1948) was chosen as representative of the neutral polyamino acids. It is the only known water-soluble member of this group. The synthetic poly-L-aspartic acid (Berger & Katchalski, 1951) and the natural poly-D-glutamic acid (Hanby & Rydon, 1946) were selected as representatives of the acidic polyamino acids. Poly-L-lysine and poly-DL-lysine (Katchalski, Grossfeld & Frankel, 1948) as