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URINARY METABOLITES OF HARMINE IN THE RAT AND THEIR INHIBITION OF MONOAMINE OXIDASE

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Abstract—Tritiated harmine hydrochloride (5 mg/kg, i.p.) was given to male rats daily for 3 days and urine was pooled. Gel filtration on Sephadex G-15 yielded 5 radioactive fractions, I–V. Fraction V was shown to be harmol by comparison with authentic harmol by gel filtration, fluorescence spectrophotometry, paper chromatography, and paper electrophoresis. Fraction IV was shown similarly to be harmine. Pre-injecting rats with ^{35}S-Na_2SO_3 yielded a radioactive fraction corresponding to III; hydrolysis of III by aryl sulfatase yielded harmol; III was therefore identified as harmol sulfate. Hydrolysis of II by β-glucuronidase yielded harmol and glucuronic acid; II was therefore identified as harmol glucuronide. Fraction I was shown to be a radioactive impurity in the injection solution. The urinary metabolites occurred in the percentages: V-11 per cent, IV-2 per cent, III-69 per cent, II-18 per cent. Rat liver mitochondrial monoamine oxidase activity, measured by the conversion of benzylamine to benzaldehyde, was inhibited 27 per cent at 10^{-5}M, V; 85 per cent at 10^{-6}M, IV; 29 per cent at 10^{-5}M, III; and 52 per cent at 10^{-6}M, II.

HARMINE, one of several alkaloids obtained from the vine, *Banisteria caapi*,[1] and other South American plants, has a broad spectrum of pharmacological actions: ganglionic blockade,[2] monoamine oxidase inhibition both *in vivo* and *in vitro*,[3] hallucinogenesis of short duration,[4, 5] tremor,[6] and a variety of cardiovascular effects,[5, 7] including bradycardia, hypotension, and potentiation of the pressor response to epinephrine.

Villeneuve and Sourkes[8] identified harmalol and harmine among the metabolites of harmaline (3,4-dihydroharmine), substantiating older reports.[9] These conversions correspond to *O*-demethylation and dehydrogenation respectively.

The present study was undertaken to identify the urinary metabolites of harmine in the rat, to determine whether harmine could be hydrogenated to harmaline, and to ascertain the capability of each metabolite to inhibit monoamine oxidase.

METHODS

Male rats of the Sprague–Dawley strain were given harmine hydrochloride hydrate (5 mg/kg + 150 μc/kg tritiated harmine hydrochloride) by intraperitoneal (i.p.) injection daily for 3 days and the urine was pooled. In some experiments, i.p. injections of ^{35}S-Na_2SO_3 (67 μc/kg) preceded the harmine by 1 hr. Tritiated and ^{35}S-containing metabolites were counted on a Nuclear Chicago Mark I liquid scintillation counter.

Gel filtration was performed with Sephadex G-15 on a 90 × 1·7 cm column.

One-ml samples, containing 2 mg of blue dextran as a high molecular weight marker, were used. Elution was made with 0·08 M formic acid, titrated to pH 3·75 with 29% aqueous NH_3. Twelve-ml fractions were collected using a Gilson fraction collector and u.v. monitor recording per cent transmission at 254 mμ. One-tenth ml was removed from each fraction for the determination of radioactivity.

Ascending paper chromatograms were developed on Whatman SG81 silica gel impregnated paper in the following systems:

System 1: *n*-butanol: acetic acid: water (3:1:1) (vvv).
System 2: isopropanol: 29% aqueous NH_3 (8:2) (vv).
System 3: isoamyl alcohol: pyridine: water (7:7:6) (vvv).

Fluorescent compounds on the paper were detected visually by use of a u.v. lamp. Radioactivity was detected by cutting sequential 2·5 mm strips from the chromatogram and placing them in scintillation vials with 10 ml of toluene containing fluors (prepared from Nuclear Chicago spectrafluor). To identify hexoses, chromatograms were dipped in a $AgNO_3$ solution (0·5 ml saturated aqueous $AgNO_3$ + 1·5 ml H_2O + 100 ml acetone) followed by alcoholic NaOH (5 ml 50% NaOH + 200 ml 95% ethanol); the brown color which developed was fixed by treating the chromatograms with 5% sodium thiosulfate.

Paper electrophoresis was done on Whatman 3M paper for 1 hr at 3000 V and 40–80 mA. For electrophoresis at low pH, 0·1 M formic acid (pH 2·4) was used as the buffer; at high pH, 0·05 M NaOH (pH 12·3) was used. Fluorescent compounds were detected visually by use of a u.v. lamp.

Enzymatic hydrolysis of products was carried out using marine mollusc β-glucuronidase-aryl sulfatase containing 20% sorbitol; the enzyme concentration was 1 mg/ml in either 0·5 M sodium acetate or 0·2 M sodium phosphate buffer (both at pH 4·1) for 2·5 or 12 hr. Phosphate buffer markedly attenuates aryl sulfatase activity.[10]

All samples were diluted with 0·1 N H_2SO_4 and fluorescence spectra were determined using an Aminco–Bowman recording spectrophotofluorometer.

Rat liver mitochondria were obtained by the method of Schneider and Hogeboom.[11] Two g of rat liver was homogenized in 10 ml of 0·25 M sucrose and centrifuged for 10 min at 700 *g*. The supernatant was removed and centrifuged for 10 min at 5000 *g*. The pellet from the second centrifugation was suspended in 1000 ml of 0·1 M potassium phosphate buffer (pH 7·2). Monoamine oxidase activity was measured by the method of Tabor *et al.*[12] Nine-tenths ml of the mitochondrial suspension was added to 0·025 ml of the appropriate inhibitor solution (water, in the case of standards; normal rat urine in the case of controls). One-tenth ml of a 1% solution of benzylamine was added and the optical density change at 250 mμ was followed with a Zeiss PMQ II spectrophotometer until a change of 0·1 to 0·2 optical density units was obtained with the standard (30–60 min at ambient temperature).

Randomly labeled tritiated harmine hydrochloride (1800 mc/m-mole) and ^{35}S-Na_2SO_3 (5 mc/m-mole) were obtained from the New England Nuclear Corp. Harmine hydrochloride hydrate and harmol hydrochloride monohydrate were obtained from the Aldrich Chemical Corp. β-Glucuronidase-aryl sulfatase in 20% sorbitol containing 2000 Fishman units[13] of β-glucuronidase and 3500 units of aryl sulfatase per mg was obtained from Pabst Biochemicals.

RESULTS

Gel filtration of 1-ml urine samples from the rats treated with tritiated harmine gave five radioactive fractions (I–V) (Fig. 1). Excitation fluorescence spectra of the fractions were determined at an emission wavelength of 419 mμ (Fig. 2). The spectrum of fraction I was indistinguishable from that of normal rat urine, while the spectra of fractions IV and V were identical to those of authentic harmine and harmol. Similarities were also observed among authentic harmine and fractions II and III; the excitation wavelength of the major harmine peak (321 mμ) was shifted slightly in the spectra of the fractions and the shoulder (355mμ) was exaggerated into a third peak.

Gel filtration of the injection solution indicated the presence of a radioactive

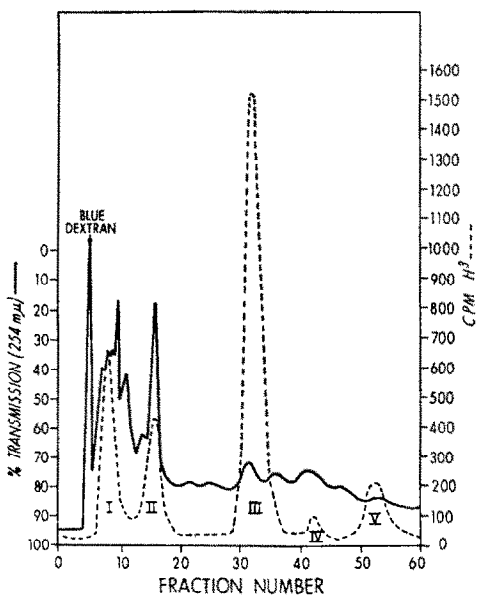

FIG. 1. Gel filtration on Sephadex G-15 of urine from rats given H^3-harmine hydrochloride and harmine hydrochloride hydrate. Blue dextran was used as a high molecular weight marker. Solid line shows per cent transmission; broken line shows CPM ^3H. Each of the five radioactive fractions (I–V) contained blue-fluorescing material.

impurity (or impurities) with the same elution volume as fraction I. Chromatography of fraction I in system 1 failed to reveal fluorescent compounds not found in normal rat urine; no distinct peak of radioactivity was found in the paper chromatogram; rather, a smearing of the radioactivity was observed. Smearing was also obtained with the radioactive impurity in the injection solution. In all probability, fraction I is this impurity and not a metabolite of harmine.

Chromatograms of fractions II, III, IV, and V, harmine, and harmol were developed (Table 1). In system 1, fractions IV, V, harmine and harmol had identical R_f's (0·78), but fractions II and III moved more slowly, with R_f's of 0·22 and 0·46 respectively. In system 2, however, fraction IV moved nearly with the solvent front (as did harmine) with an R_f of 0·97, whereas fraction V and harmol were retarded slightly ($R_f = 0·75$).

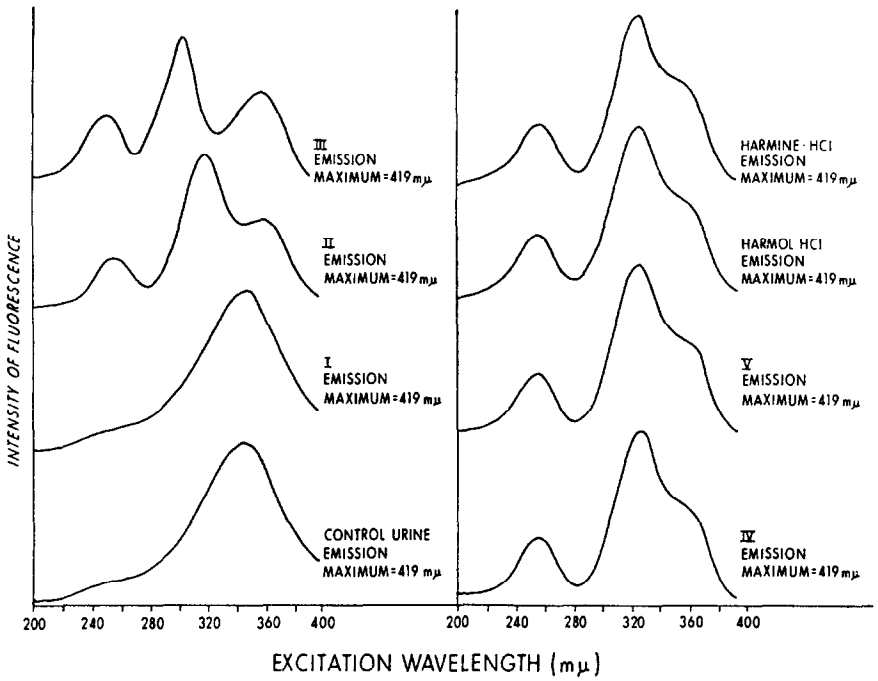

FIG. 2. Fluorescence spectra in 0·1 N H_2SO_4 of normal rat urine, of fractions I–V from rats given H^3-harmine hydrochloride and harmine hydrochloride hydrate, of harmol hydrochloride, and of harmine hydrochloride. In all cases, the emission wavelength was 419 mμ. The spectra of fraction I and normal rat urine were identical, as were those of fractions IV, V, harmine, and harmol.

TABLE 1. R_f'S OF HARMINE AND METABOLITES

	System 1	System 2
Harmine	0·78	0·97
Harmol	0·78	0·75
Fraction V	0·78	0·75
Fraction IV	0·78	0·97
Fraction III	0·46	
Fraction II	0·22	

An amount of fraction V containing radioactive metabolite insufficient to yield visible fluorescence when spotted on Whatman SG81 paper was mixed with 1 ml of harmol hydrochloride monohydrate (0·1 mg/ml) and subjected to paper chromatography in systems 1 and 2, and gel filtration. The paper and gel filtration fractions were analyzed for both fluorescence (visually with a u.v. lamp) and radioactivity. The fluorescence from the added harmol was congruent with the radioactivity from fraction V in both chromatographic systems. In gel filtration on Sephadex G-15, both harmol and fraction V were eluted at fraction No. 53.

Electrophoresis of harmol and fraction V was performed. Since harmol is amphoteric, containing both a phenolic group and an aromatic tertiary amine group, the molecular charge and hence its direction of migration are a function of pH. Both harmol and fraction V carried a positive charge at low pH and a negative charge at

high pH. Harmol and fraction V were indistinguishable on the basis of paper chromatography, gel filtration, paper electrophoresis, and fluorescence spectrophotometry.

Fraction IV was mixed with harmine hydrochloride hydrate in a manner identical to that described above for fraction V and harmol and subjected to paper chromatography and gel filtration. Fraction IV and harmine behaved identically in both paper chromatographic systems 1 and 2. In gel filtration, both harmine and fraction IV were eluted at fraction No. 39.

Electrophoretically, harmine, which contains only a basic functional group, moved toward the negative pole at low pH and not at all at high pH; fraction IV behaved similarly. By these criteria, fraction IV was indistinguishable from harmine.

To test the possibility that fraction III might be an ethereal sulfate, ten rats were given ^{35}S-sodium sulfite by i.p. injection. One hr later, half the animals received harmine hydrochloride hydrate (10 mg/kg i.p.); 2 hr after the last injection urine was collected and pooled according to group. One ml of urine from each group was subjected separately to gel filtration; the u.v. absorption at 254 mμ and the radioactivity of each fraction were determined. The urine from the harmine-treated animals contained a peak of radioactivity that did not appear in the controls and which corresponded to fraction III. Paper chromatography of this radioactive fraction in system 1 confirmed it as identical to fraction III. The presence of ^{35}S in fraction III implied that it was an ethereal sulfate. Accordingly, enzymatic hydrolysis with β-glucuronidase-aryl sulfatase was carried out. The radioactive fraction was divided into four parts; one of the following solutions was added to each: 0·5 M sodium acetate (pH 4·1) containing 1 mg/ml β-glucuronidase aryl sulfatase, 0·2 M sodium phosphate (pH 4·1) containing 1 mg/ml β-glucuronidase-aryl sulfatase, and one of each buffer without enzyme to serve as controls. Phosphate buffer has been shown to inhibit marine mollusc aryl sulfatase.[10] All four samples were incubated at 37° for 12 hr; at the end of this time, chromatograms of 0·01 ml aliquots of each were developed in system 1. Change in the migration of radioactivity or of fluorescent compounds from that of the fraction prior to treatment was noted only in the enzyme-treated fraction in acetate buffer. Enzymatic hydrolysis in acetate buffer converted about 75 per cent of the radioactive fraction to a nonradioactive fluorescent compound, which was identical to harmol by the same tests used to identify the metabolite in fraction V. Simultaneously, a peak of ^{35}S activity corresponding to ^{35}S-sulfate ($R_f = 0{\cdot}15$) appeared. This indicates that fraction III is harmol sulfate.

Fraction II was treated with β-glucuronidase-aryl sulfatase in a manner identical to that described above for the ^{35}S fraction. In this case, however, hydrolysis was essentially complete in 2·5 hr; 0·2 M sodium phosphate buffer did not inhibit the enzyme. Chromatography of the hydrolysate, the untreated fraction II, the enzyme solution, and glucuronic acid in system 3 was carried out. The hydrolyzed fraction contained glucuronic acid and sorbitol, which were absent in the untreated sample; sorbitol is present in the commercial enzyme preparation. The fluorescent product which appeared after enzymatic hydrolysis of fraction II was also identical to harmol in the systems used to identify harmol as the metabolite in fraction V. This indicates that fraction II is harmol glucuronide.

The same four compounds, harmol, harmine, harmol sulfate, and harmol glucuronide, were found in the bile of rats treated with harmine.

The amount of metabolite in fractions II and III was estimated by hydrolyzing each

TABLE 2. MAO INHIBITION BY HARMINE AND METABOLITES (% INHIBITION ± S.E.)*

Fraction	Metabolite	10^{-7}M	10^{-6}M	10^{-5}M
II	Harmol glucuronide	0±1†	52±3§	92±4§
III	Harmol sulfate		0±3†	29±6‡
IV	Harmine	1±9†	86±6§	97±3‖
V	Harmol		0±5†	27±4‡

* Inhibition by controls was insignificant. Three determinations were made at each concentration.
† Not significant ($P > 0.05$).
‡ $P < 0.05$.
§ $P < 0.01$.
‖ $P < 0.001$.

with β-glucuronidase-aryl sulfatase and measuring the harmol formed by quantitative spectrophotofluorometry. Quantitation of the harmine and harmol present in fractions IV and V was also obtained by fluorescence measurements. Solutions of each fraction were prepared so that the addition of 0·025 ml to 1 ml of the monoamine oxidase assay solution yielded metabolite concentrations of 10^{-7}, 10^{-6}, or 10^{-5}M. Three determinations were made at each concentration; the results are shown in Table 2. Harmine inhibited the monoamine oxidase 86 per cent at 10^{-6}M; harmol inhibited the enzyme 27 per cent at 10^{-5}M. Harmol sulfate was about as potent as harmol, but harmol glucuronide was considerably more potent than harmol, inhibiting the monoamine oxidase 52 per cent at 10^{-6}M.

DISCUSSION

Pletscher *et al.*[9] and, more recently, Villeneuve and Sourkes[8] reported the conversion of harmaline to harmalol and harmine in the rat. In the present study, harmol, harmol sulfate, and harmol glucuronide were identified as the urinary metabolites of harmine.

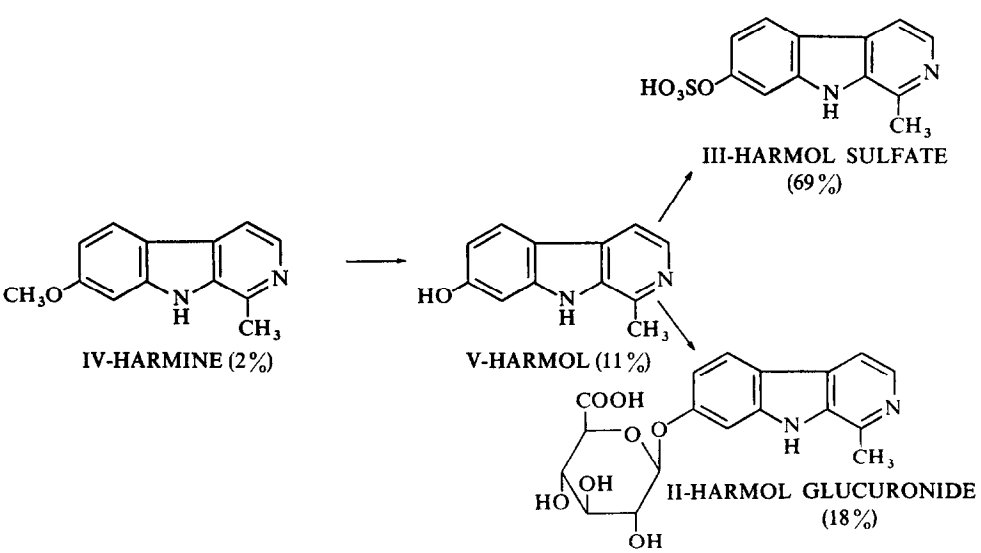

FIG. 3. Proposed pathway for harmine biotransformation in the rat, showing the percentages of total metabolite found in the urine.

No harmaline derivatives were found, indicating that although harmaline may be dehydrogenated to harmine *in vivo*, hydrogenation of harmine to form harmaline does not occur. The *O*-demethylation which occurs in the metabolism of harmaline is observed with harmine, however.

In view of these observations, a pathway for harmine biotransformation can be proposed; the percentages of total urinary metabolites are shown for each compound (Fig. 3).

The monoamine oxidase inhibition produced by harmol and harmine in rat liver mitochondria approximated those values reported for guinea pig liver mitochondria.[3] Conjugation of harmol with glucuronic acid resulted in a restoration of monoamine oxidase inhibitory potency to a level only slightly less than that of harmine, whereas conjugation with sulfuric acid did not result in a significant change in activity from that of harmol.

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