Synthesis and Metabolism of Labeled Acetohydroxamic Acid, a Urease Inhibitor[1] (35318)

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Ammonia toxicity contributes to hepatic coma and, under fasting circumstances, most of the ammonia produced in the body derives from hydrolysis of urea in the gastrointestinal tract (1–3). Conventional treatment for hepatic coma includes protein restriction, enemas, and the use of broad-spectrum antibiotics. These measures presumably inhibit the production of ammonia from urea in the gut by decreasing the amount of substrate available and suppressing bacterial urease activity, but the effects are incomplete (4). Therefore, more specific methods of inhibiting urease activity have been examined and tentatively applied as adjuncts in the treatment of hepatic coma (5, 6), especially when ammonia and urea concentrations in the blood are high. In particular, acetohydroxamic acid (AHA), a specific inhibitor of urease activity, has been considered to have potential therapeutic application (6–10).

In vitro, AHA efficiently inhibits urease activity from the jack bean as well as that present in mucosa or feces from the human gastrointestinal tract (4, 7). *In vivo*, AHA has a low toxicity and inhibits ureolysis in mice (9), decreases ammonia concentration in the sheep rumen (11), and prevents the increase in blood ammonia concentration which otherwise follows intravenous administration of urea to dogs with Eck fistulas (6). Preliminary observations suggested that AHA may decrease blood ammonia concentration in patients in hepatic coma (6).

The data available regarding the metabolism and excretion of AHA are scanty, although some studies have been carried out in mice (12) and dogs (6). We have investigated the absorption, excretion, and metabolic fate of AHA in the dog and in patients with hepatic cirrhosis. To this end, ^{3}H- and ^{14}C-labeled AHA were synthesized because the customary spectrophotometric technique for measuring AHA is nonspecific and cannot be used to detect metabolic derivatives. In addition, the availability of ^{3}H-labeled AHA would permit concurrent studies utilizing ^{14}C-labeled urea.

Materials and Methods. Synthesis of radioactive AHA. Hydroxylamine hydrochloride (250 μmoles) was dissolved in 250 μl of 1 *N* sodium hydroxide (250 μmoles), and 5 mCi of ^{3}H-labeled acetic anhydride (Tracerlab, Waltham, Mass.), diluted with carrier acetic anhydride to 125 μmoles (11.75 μl), was added. The reaction mixture was allowed to stand overnight and then was reduced to dryness with an air stream; the residue was dissolved in ethanol–chloroform, 1:1. The labeled AHA was isolated by preparative thin-layer chromatography on silicic acid plates, using a solvent system of *n*-butanol–glacial acetic acid–water, 8:2:2. The region of the plate having a mobility corresponding to the AHA standards, which were applied on each side of the reaction mixture, was transferred to a sintered glass funnel and eluted three times with water and once with ethanol. The ^{14}C-labeled AHA was prepared similarly from 1-^{14}C-labeled acetic anhydride. Nonradioactive AHA, prepared by this method and crystallized from ethyl acetate, had a melting point of 68° (reported 69 to 88° [13, 14]).

Nonradioactive AHA used in these studies was synthesized and generously supplied by Dr. W. H. Fishbein, Armed Forces Institute of Pathology, Washington, D.C. Details of preparation of the compound and proof of

[1] This investigation was supported in part by Research Grant AM-6908 from the National Institutes of Health, Public Health Service, and by the Rogers Foundation.

purity have been published (15).

Pharmacologic studies. To investigate absorption and excretion of AHA and to determine the relative stabilities of the ^{3}H and ^{14}C labels, a mixture of AHA-^{14}C (5 to 10 μCi) and AHA-^{3}H (20 to 40 μCi) was given to dogs by vein (animals 1 and 3) or by mouth (animals 2 and 4). Urine was collected 12 and 24 hr later via an indwelling bladder catheter; feces also were collected for 24 hr.

To investigate absorption and excretion of AHA in man, studies were made in four patients (three men and one woman, ages 46 to 65 years) with cirrhosis complicated by chronic encephalopathy (16). The diagnosis was evident on clinical examination, was supported by abnormalities of hepatic function, and was confirmed by both liver biopsy and electroencephalography. All patients were studied in a metabolic unit to which they were admitted 3 days prior to the investigation, and all received a constant diet providing a protein intake of 30 g daily. AHA (2 mmoles/kg and containing 50 μCi of ^{3}H) was given by intragastric tube after the patient had fasted overnight. The compound was dissolved in 5% dextrose and the solution was adjusted to pH 7.3 (with sodium bicarbonate). Patients 1 and 2 were studied twice. Measurements of hematologic indices (hemoglobin, leukocyte total and differential counts, and platelet count), renal function (urinalysis, blood urea and plasma creatinine concentrations, and creatinine clearance), and hepatic function (concentrations of serum bilirubin and proteins, sulfobromophthalein retention, and serum activities of glutamic-oxaloacetic transaminase and alkaline phosphatase) were performed immediately before and within 5 days after the administration of AHA. Normal individuals were not studied because AHA currently is restricted to investigational use in patients with cirrhosis and encephalopathy.

Analytical methods. ^{3}H and ^{14}C radioactivity was determined by liquid scintillation spectroscopy, with corrections for quenching and channel spillover made by external standardization. Urine or serum (1 ml) was mixed with 9 ml of absolute ethanol, heated, and centrifuged for 30 min at 4000 rpm to remove precipitated protein. An aliquot (1.0 ml) of the supernatant solution was counted in a dioxane-based scintillation mixture. Urine specimens from three patients and one dog were freeze-dried, and the residue was suspended in absolute ethanol. The chemical distribution of radioactivity in the ethanol was determined by zonal scanning (17) using silicic acid plates and a solvent system of *n*-butanol saturated with 10% NH_4OH (R_F: AHA, 0.2; acetamide, 0.5).

Results. Synthesis, radiopurity, and label stability. A zonal scan of AHA-^{3}H (Fig. 1) demonstrated radiopurity of >97%, and a similar chromatogram was obtained with AHA-^{14}C. Yields from the radiosynthesis procedure were low (^{3}H, 50%; ^{14}C, 12%). Zonal scanning revealed no radiodecomposition after the material had been stored in water at 4° for 20 months. Stability of the ^{3}H label was evident from the constant $^{3}H/^{14}C$ ratio in all urine specimens after compounds with each label had been administered simultaneously to dogs (Table I). In addition, zonal scans of urine specimens from dogs (not shown) indicated that all ^{3}H and ^{14}C radioactivity had a mobility identical to that of AHA; no other radioactive metabolites were detected.

Absorption and excretion of AHA in dogs. AHA was rapidly absorbed when given orally (the label quickly appeared in the urine). Excretion of the label in the urine also was rapid when the compound was given by vein

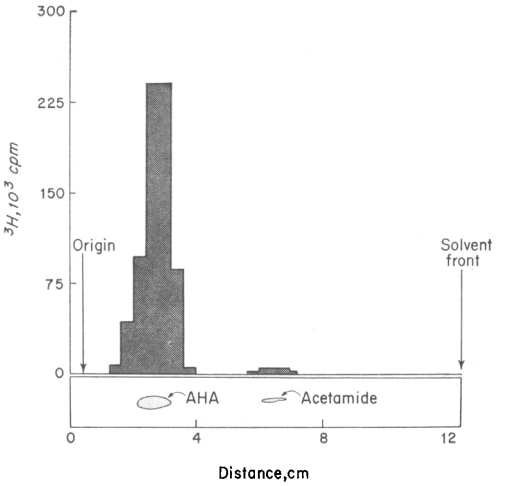

FIG. 1. Zonal scan of synthetic AHA-^{3}H (silicic acid, *n*-butanol saturated with 10% (w/v) NH_4OH).

TABLE I. Urinary Excretion of Radioactivity After Administration of ^{3}H- and ^{14}C-Labeled AHA.

Dogs	Route of admn	Urine collection (hr)	Recovery (%)		Ratio, $^{3}H/^{14}C$[a]
			^{3}H	^{14}C	
1	Intravenous	12	52.6	55.8	0.94
2	Oral	12	51.0	52.5	0.97
3	Intravenous	24	78.0	79.5	0.98
4	Oral	24	91.6	90.7	0.98

[a] The $^{3}H/^{14}C$ ratio of administered AHA was adjusted to 1.0.

(Table I). More than half the radioactivity administered by either route was recovered in the urine within 12 hr, and up to 90% of the dose was recovered within 24 hr; no radioactivity was detectable in the feces during this time.

Absorption and excretion of labeled AHA in patients with cirrhosis. AHA was rapidly absorbed after being given by mouth; radioactivity was detected in the blood within 3 hr and thereafter decreased exponentially (Fig. 2). In most instances, radioactivity appeared in the urine within 6 hr (Table II) and thereafter urinary excretion decreased exponentially (Fig. 3). Zonal scan of an alcoholic extract of freeze-dried urine indicated that the radioactivity appeared to be excreted largely as AHA, with $<5\%$ having the mobility of acetamide (Fig. 4). Radioactivity was not present in feces during the 48 hr of the study.

The slower excretion of AHA in patients with cirrhosis than in healthy dogs was presumably due to the impairment of renal circulation associated with the advanced liver disease (19) which was present in all patients (Table II). The presence of ascites, with greater body distribution of AHA, may have contributed in two patients. The two patients (nos. 3 and 4) with most severely impaired renal function, in whom the lowest creatinine clearances were associated with increased blood urea concentrations, showed slower excretion of AHA. In the two patients with better renal function, 50% of the dose administered was recovered from the urine in 48 hr. Extrapolation of the data from the four studies in these two patients indicated that between 80 and 90% of AHA would be excreted in the urine within 4 days of administration.

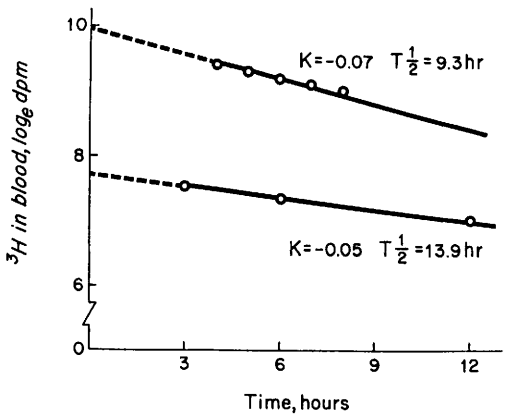

FIG. 2. ^{3}H radioactivity in blood of patients with cirrhosis after administration of AHA-^{3}H.

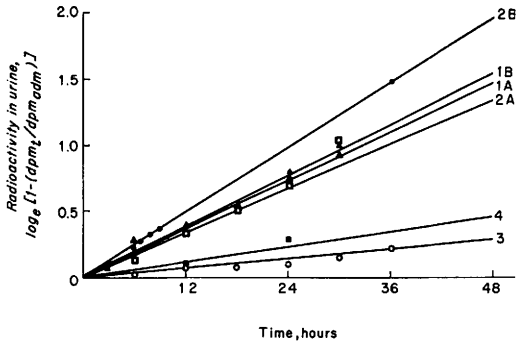

FIG. 3. Cumulative ^{3}H radioactivity excretion in urine by cirrhotic patients after injection of AHA-^{3}H. The numbers refer to the subjects and studies (see Table II). The use of a logarithmic transform for the plotting of cumulative excretion data was proposed by Lindstedt and Norman (18).

The $T_{½}$ estimates based on decrease in blood radioactivity and on urinary excretion were in fair agreement (patient 1, study B:

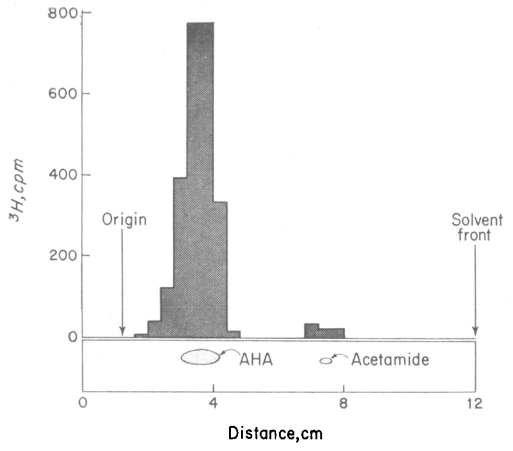

FIG. 4. Zonal scan of ^3H radioactivity in urine from patients with cirrhosis after administration of AHA-^3H. The substance with the mobility of AHA gave a red color when sprayed with ferric chloride, providing further evidence that it was AHA.

blood, 13.9 hr; urine, 12.7 hr; patient 2, study A: blood, 9.3 hr; urine, 11 hr). The apparent volume of distribution of AHA, calculated by extrapolating blood radioactivity data to zero time, was 50.9 liters (patient 1, study B) and 30.6 liters (patient 2, study A) and consistent with ready diffusion of AHA into the interstitial spaces.

Side-effects. Although hydroxamates have had no effect on a variety of enzymes other than urease, the compounds have been implicated as carcinogenic and teratogenic agents. Our earlier studies with AHA in dogs and in patients with end-stage hepatic coma failed to show side-effects (6). In the present investigation, AHA given to patients as a single dose by mouth caused nausea in two; one of these vomited. Blood hemoglobin concentration decreased by more than 2 g/100 ml in two patients. There was no significant change in leukocyte total and differential counts. A transient decrease in platelet count occurred in all but one instance but never approached pathogenic levels. Results of urinalysis, tests of renal function, and tests of hepatic function were unaltered.

Discussion. Between 80 and 90% of the administered AHA was accounted for by urinary excretion as unchanged AHA. This observation, together with our other data, indicates that the absorption, distribution, and metabolism of AHA resemble those of urea, its competitive substrate. Less than 10% of the excreted radioactivity appeared as acetamide, although in mice approximately 60% of the dose of intraperitoneally administered AHA can be recovered from the urine in the following 48 hr (12), 80% as AHA and the remainder as acetamide. Excretion of the acyl moiety of hydroxamic acids as its corresponding amide is an accepted metabolic pathway in mammalian systems, but this pathway appears much less significant quantitatively in the dog and in cirrhotic man than in the mouse. An additional metabolic pathway for acetohydroxamic acid, neither confirmed nor excluded by our own or other *in vivo* studies, might involve degradation to carbon dioxide and water by bacterial enzymes in the gut or by tissue enzymes.

Summary. ^{14}C- and ^3H-labeled acetohydroxamic acid were synthesized and purified by preparative thin-layer chromatography.

TABLE II. Kidney Function and Kinetics of Urinary Excretion in Cirrhotic Patients.

Patient	Study	Creatinine clearance (ml/min)	Cumulative urinary excretion of radioactivity (% of dose administered) 6 hr	12 hr	24 hr	48 hr	Slope[a] (hr^{-1})	$T_{½}$[a] (hr)
1	A	86	13.0	16.0	26.0	50.0	0.054	12.9
	B		7.3	13.3	22.2	50.0	0.055	12.7
2	A	80	4.5	12.3	25.0	45.0	0.063	11.0
	B		11.6	15.4	26.7	59.0	0.038	18.0
3		25	0.2	0.5	1.8	6.0	0.008	80.9
4		21	—	0.4	2.7	10.0	0.025	27.8

[a] Slope and $T_{½}$ were calculated from excretion data for 48 hr.

When these compounds were given simultaneously to dogs, the $^{3}H/^{14}C$ ratio remained constant in urine, indicating that the ^{3}H label was stable *in vivo*. In dogs, AHA was rapidly absorbed when given orally; when given by this route or intravenously, 80 to 90% of the administered radioactivity was recovered in the urine in 24 hr, predominantly as unmetabolized AHA. AHA was given by mouth to patients with cirrhosis and encephalopathy; radioactivity appeared promptly in the blood and urine and decreased exponentially. Urinary excretion was slower than in dogs, probably because of concomitant impairment of renal function. Less than 10% of urinary radioactivity was present as acetamide in dogs or patients. AHA was not excreted in the feces during the duration of the experiment. Side-effects of AHA given to patients included gastrointestinal symptoms and transient decrease of the blood platelet count.

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Received Sept. 23, 1970. P.S.E.B.M., 1971, Vol. 136.