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THE METABOLIC FATE OF 1,4-DIHYDROXYPHTHALAZINE-1-^{14}C*

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Abstract—Radioactive 1,4-dihydroxyphthalazine was synthesized and its metabolic fate studied. Isotope dilution studies showed that 30–34% was excreted unchanged and 50% was conjugated with glucuronic acid. N-Methylation accounted for 3·5% of the dose in rats but not in rabbits. The major metabolite, 1,4-dihydroxyphthalazine-O-glucuronide was isolated and characterized. 1,4-Dihydroxyphthalazine was found to be relatively nontoxic, the minimal lethal dose being greater than 320 mg/kg.

DRUGS derived from hydrazine have been used in the treatment of tuberculosis, polycythemia, hypertension, and mental depression. Many of these hydrazine compounds, however, have the serious drawback of toxic side effects. Peripheral neuritis due to isoniazid therapy has been reported by Biehl and Vilter,[1] and hepatitis is associated with iproniazid.[2–3] Hydrazine and some of its derivatives have been found to produce fatty degeneration of the liver and convulsions (McKennis *et al.*[5] and Yard and MacKennis[6]). The toxicity of hydrazides has been related to the release of hydrazine from them *in vivo* by McIsaac and Williams,[7] and it has been suggested that the formation of hydrazones *in vivo* with consequent pyridoxal deficiency plays an important role in the toxicity of these compounds.[8] The toxicity of many hydrazine drugs in use has led to the study of cyclic hydrazine compounds, which are less toxic, as possible therapeutic agents. Since little is known about the metabolic fate of phthalazine derivatives, 1,4-dihydroxyphthalazine-1-^{14}C was synthesized and its metabolic fate studied.

EXPERIMENTAL PROCEDURE

Compounds

1,4-Dihydroxyphthalazine-1-^{14}C (m.p. 335°, sp. act. 375 μc/g) was synthesized by condensation of phthalic-7-^{14}C anhydride with hydrazine.

1,4-Dihydroxy-N-methylphthalazine (m.p. 230°) was prepared by methylation of the sodium salt of 1,4-dihydroxyphthalazine.[9] O-N-Diacetyl-1,4-dihydroxyphthalazine (m.p. 139 to 140°) was prepared by acetylation of 1,4-dihydroxyphthalazine.[10] N-Acetyl-1,4-dihydroxyphthalazine (m.p. 175°) was prepared by alcoholysis of O-N-diacetyl-1,4-dihydroxyphthalazine.[10] Phthalic acid was obtained commercially and its dimethyl ester prepared by methylation.

Animals

Albino rats of 200 g and rabbits of Dutch or albino strain of 2·5–3 kg were used. Compounds were administered in aqueous solution by stomach tube. Animals were

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fed on a standard diet with water *ad libitum* and kept in metabolism cages while under experiment.

Analytical methods

Glucuronic acid was determined by the method of Paul.[11]

Chromatographic methods

For the detection of metabolites in urine, descending chromatography with Whatman No. 1 paper was used. The solvents, R_f values, and color reactions of reference compounds employed are given in Table 1. Radioactive chromatograms were scanned.

TABLE 1. R_f VALUES AND COLOR REACTIONS OF COMPOUNDS RELATED TO 1,4-DIHYDROXYPHTHALAZINE

Solvent systems used were: (A) propanol-1-ol– conc. NH_3 (8 : 2); (B) *n*-butanol–acetic acid–water (4 : 1 : 5); (C) acetonitrile–water (3 : 1). The sprays used for detecting compounds on paper were Folin Ciacolteau, methyl red in borate buffer (pH 8), and silver nitrate (1 N).

Compound	R_f values in solvent			Color of spots on paper with		
	A	B	C	Folin Ciacolteau	Methyl red	$AgNO_3$
1,4 Dihydroxyphthalazine	0·19	0·82	0·77	Blue		
1,4 Dihydroxyphthalazine-O-glucuronide	0·19	0·82	0·32	Blue		
Phthalic acid	0·59	0·92			Pink	
N-Methyl-1,4 dihydroxyphthalazine	0·19	0·44	0·86			Black
N-Acetyl-1,4 dihydroxyphthalazine	0·20	0·80	0·18	Blue		

Measurement of radioactivity

Measurements were carried out on solid samples of 'infinite thickness' on nickel planchets with an end-window counter tube, the background of which was 20 cpm. The specific activities were determined by comparison with a stable polymer reference. A sample of 4 cm^2 containing 0·1 μc ^{14}C/g substance gave approximately 270 cpm.

Urine and tissues

The radioactivity of urine and tissues was measured after drying directly on planchets under infrared lamps.

Isotopic dilution methods

For the estimation of metabolites by isotopic dilution, urines were collected for 24 hr. The volume used depended upon the substance estimated. 1,4-Dihydroxyphthalazine (0·5 g) was added to one third volume of urine and the pH adjusted to 6. By gentle warming the 1,4-dihydroxyphthalazine was dissolved and allowed to stand for 2 hr to equilibrate. The 1,4-dihydroxyphthalazine subsequently crystallized on standing at 5° overnight. The compound was recrystallized to contant specific activity, melting point, and mixed, m.p. 335°.

1,4-*Dihydroxy-N-methylphthalazine.* This was dissolved (0·25 g) in one fifth volume of urine by dilution to 50 ml with water. Reinecke salt (0·4 g) in ethanol was added and the corresponding reineckate crystallized out. The reineckate was then recrystallized from aqueous alcohol to constant specific activity, melting point and mixed, m.p. 264°.

N-Acetyl-1,4-dihydroxyphthalazine. This was dissolved (0·5 g) in one fifth volume of urine, but only 1,4-dihydroxyphthalazine could be recovered, since deacetylation rapidly took place.

Phthalic acid. This was dissolved (0·25 g) in one fifth volume of urine by dilution to 60 ml with water and allowed to equilibrate for 2 hr. Phthalic acid recovered was contaminated with 1,4-dihydroxyphthalazine, so it was methylated with diazomethane to give the dimethyl ester. Traces of contaminating dihydroxyphthalazine converted to the N-methyl derivative were then removed by forming the reineckate. Excess reinecke salt was removed by the addition of silver nitrate and the insoluble silver reineckate filtered off. The activity of the phthalic acid dimethyl ester recovered from the filtrate was then assayed.

RESULTS

Toxicity of 1,4-dihydroxyphthalazine

Ten rats were given 1,4-dihydroxyphthalazine orally in doses ranging from 100 to 320 mg/kg. All the animals survived, and no toxic effects were noted.

Rate of excretion and distribution in tissues of administered 1,4-dihydroxyphthalazine-1-^{14}C

The rate of excretion of metabolites in the urine after administration of 1,4-dihydroxyphthalazine-1-^{14}C (5 mg to rats and 10 mg to rabbits) was measured by the activity present. In both species 70 $\pm$ 5% of the activity appeared in the urine within 24 hr and 90% within 48 hr. Distribution of activity in the tissues of rats killed 24 hr after administration is shown in Table 2.

TABLE 2. EXCRETION OF RADIOACTIVITY IN THE URINE AND DISTRIBUTION IN THE TISSUES 24 HR AFTER ADMINISTRATION OF 5 mg OF 1,4 DIHYDROXYPHTHALAZINE-1-^{14}C TO A RAT

Tissue	Specific activity (μc/g)	Percentage of dose
Blood	0·01	3·77
Heart	0·0012	0·011
Liver	0·0021	0·23
Kidney	0·005	0·02
Spleen	0·002	0·054
Skeletal muscle	0·0031	0·054
Stomach and intestine	0·0042	1·40
Lungs	0·0031	0·054
Total percentage in tissues		5·6
Urinary excretion		72·5
Total activity accounted for		78·1

Identification of metabolites

Scanning radioactive chromatograms obtained by chromatographing urine in solvents A and B disclosed a major peak of radioactivity coincident with the R_f of 1,4-dihydroxyphthalazine. Separation of 1,4-dihydroxyphthalazine and its glucuronide could be obtained only in solvent C. The presence of 1,4-dihydroxyphthalazine glucuronide in the urine of rabbits dosed with ^{14}C-1,4-dihydroxyphthalazine was noted.

Quantitation of metabolites

Glucuronide formation was estimated by a colorimetric method[11] in three rabbits. Triplicate estimations of glucuronic acid excreted by each of the three animals on four control days were made. The increase above this base level during the 24-hr period following administration of 0·64 g 1,4-dihydroxyphthalazine to each animal showed that 50% of the dose was excreted in conjugation with glucuronic acid.

Other metabolites of 1,4-dihydroxyphthalazine were estimated by isotope dilution methods. Triplicate estimations showed that in rabbits 30 ± 3% and in rats 45 ± 4·8% was excreted unchanged. 1,4-Dihydroxy-N-methylphthalazine was found in rat urine, 3·5% of the dose, but not in rabbit urine. Phthalic acid was estimated as being present in rat urine to the extent of 7·3% of the dose; however, since there was considerable difficulty in removing contaminating activity this figure should be interpreted with caution. Certainly not more than 7·3% of 1,4-dihydroxyphthalazine is broken down to phthalic acid and probably much less.

Isolation of metabolites

Four rabbits were dosed with 1 g 1,4-dihydroxyphthalazine each and the 24-hr urines collected and pooled. The lead acetate fractionation method of Kamil *et al.*[12] was employed. The urine was adjusted to pH 3–4 with glacial acetic acid. A fifth of the volume of normal lead acetate solution was added and the mixture centrifuged at 2,000 rev/min for 10 min. The sediment was discarded and the supernatant fluid adjusted to pH 8·5 with concentrated ammonia. The mixture was centrifuged for 10 min at 2,000 rev/min. The supernatant was discarded and the deposit washed with distilled water and resuspended in 200 ml water and H_2S gas bubbled through it for 1 hr. The black precipitate was filtered off and the filtrate concentrated *in vacuo* at 40–45° to a volume of 50 ml. On standing, a precipitate formed and was filtered off. This proved to be unchanged 1,4-dihydroxyphthalazine, m.p. and mixed m.p. 335°. The filtrate was further concentrated to 20 ml and stood at 10° for 48 hr; a precipitate formed and was filtered off and recrystallized from aqueous alcohol to yield 1,4-dihydroxyphthalazine-O-glucuronide as a white crystalline compound, m.p. 175–180°, α_D^{20} — 59 in 0·1 N NaOH (C, 1). (Found: C, 49·94; H, 4·36; N, 8·48. $C_{14}H_{14}O_8N_2$ requires C, 49·73; H, 4·14; N, 8·28.)

Incubation of the glucuronide with β-glucuronidase at 37° in acetate buffer (pH 4·5) yielded 1,4-hydroxyphthalazine.

The triacetyl derivative of the methyl ester of 1,4-dihydroxyphthalazine-O-glucuronide was prepared, and it formed white crystals, m.p. 107–110° α_D^{20} — 6 in $CH Cl_3$ (C, 1). (Found: C, 52·93; H, 4·62; N, 6·18. $C_{21}H_{22}O_{11}N_2$ requires C, 52·72; H, 4·60; N, 5·86.)

DISCUSSION

The structure of 1,4-dihydroxyphthalazine has been the subject of some discussion, the three tautomeric forms, I, II, and III all being possibilities (see Fig. 1). Drew and Hatt[10] have suggested that I represents the most likely form but Rowe and Peters[9] have concluded that III represents the structure in neutral or acid, and II that in alkaline solutions.

In mammals phenolic compounds are readily conjugated and excreted as glucuronides. Quinones tend to be reduced to the corresponding dihydric phenols first and then conjugated with glucuronic or sulfuric acid.[13] The excretion of a glucuronide as the major metabolite would therefore be expected, irrespective of the structure of 1,4-dihydroxyphthalazine administered.

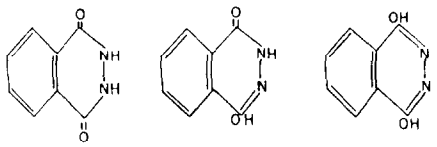

FIG. 1. 1,4-Dihydroxyphthalazine may exist in three tautomeric forms.

Heterocyclic nitrogen compounds are known to be N-methylated to a small extent in the body; for example, isoquinoline is methylated to N-methylquinolinium hydroxide.[14, 15] This N-methylation has been reported to occur in some species but not in others. N-Methyl-1,4-dihydroxyphthalazine was found in rat but not in rabbit urine.

Acetylation of 1,4-dihydroxyphthalazine leads to several different derivatives according to the conditions employed, and many misleading statements regarding these compounds have appeared in the literature. The position now appears to have been clarified.[16] Our attempts to study the possible acetylation of 1,4-dihydroxyphthalazine *in vivo* were frustrated by the ease with which the acetyl derivatives were hydrolyzed. It would seem reasonable, therefore, to conclude that should acetylation take place in the body the product would be rapidly hydrolyzed in the urine.

1,4-Dihydroxyphthalazine was relatively nontoxic, the minimal lethal dose being greater than 320 mg/kg. This is not surprising considering that little or no hydrazine, which is usually responsible for toxicity,[7] was found to be liberated during metabolism.

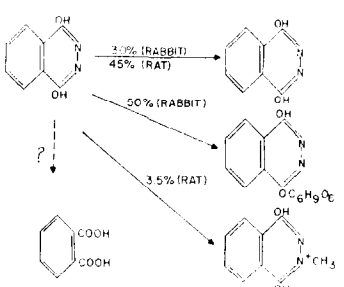

FIG. 2. 1,4-Dihydroxyphthalazine is excreted in urine, partly unchanged, partly conjugated with glucuronic acid and to a small extent as a N-methyl derivative. There are some species differences.

The metabolic fate of 1,4-dihydroxyphthalazine is represented in Fig. 2. The only species differences found were that excretion of unchanged compound was lower in rabbits than in rats and, conversely, that glucuronide conjugation was higher in rabbits than in rats, and that N-methyl-1,4-dihydroxyphthalazine was found in rat and not in rabbit urine.

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