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Disposition of the Hypolipidaemic Agent, 2,3-Dihydrophthalazine-1,4-dione, in Sprague Dawley Rats

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Abstract—The disposition of [^{14}C]2,3-dihydrophthalazine-1,4-dione, a potent hypolipidaemic agent, has been determined after both intravenous and oral administration. Both the routes of administration afforded multi-exponential disposition with an estimated $t_{\frac{1}{2}}$ of approximately 75 h. After oral administration, the drug was observed to be absorbed rapidly from the intestine and distributed quickly to all tissues of the body. A large quantity of the ^{14}C-radioactivity was found in the skin and carcass. Approximately 35% of the administered radioactivity was excreted in urine after oral administration and 11% in the faeces. Approximately 66% of the radioactivity excreted in urine was the parent drug. There was evidence of an additional metabolite which accounted for 28% of the urinary radioactive excretion. The parent drug has little serum protein binding, is highly water soluble, and is probably taken up by cells by passive diffusion.

A series of 2,3-dihydrophthalazine-1,4-diones was shown to afford potent hypolipidaemic activity in rodents (Murthy et al 1986). Both serum cholesterol (64%) and triglyceride (54%) levels were significantly reduced in rats at an oral dose of 20 mg kg^{-1} day^{-1} of 2,3-dihydrophthalazine-1,4-dione after 8 weeks (Hall et al 1988). This agent was shown to be active in diet-induced hyperlipidaemic states in mice (Murthy et al 1986). Rat tissue lipids, e.g. liver, small intestine, and aorta were reduced after treatment with the drug at 20 mg kg^{-1} day^{-1} for 8 weeks. Cholesterol levels of the VLDL and LDL were significantly reduced in rats with a 112% increase in HDL cholesterol. Regulatory enzymes of the de-novo synthesis of lipids in the liver and small intestinal mucosa were inhibited significantly in-vivo by the drug. Preliminary studies in rodents suggested that the drug was safe for therapeutic use (Hall et al 1988). A better understanding of the drug's disposition in the body and metabolism appeared appropriate. Therefore, the drug was radiolabelled and its disposition was examined in rats.

Materials and Methods

Source of compounds

2,3-Dihydrophthalazine-1,4-dione was purchased from Aldrich Chemical Co. The radiosynthesis of 2,3-dihydrophthalazine-1,4-dione [1,4-^{14}C] was carried out as follows. [Carbonyl-^{14}C]phthalic anhydride (27·0 mg, 10·0 mCi, 0·18 mmol) was dissolved in 1·0 mL of absolute ethanol and 9·0 mg (0·18 mmol) of hydrazine hydrate was added in one portion by syringe. A precipitate formed immediately which dissolved upon heating. The reaction was stirred at reflux for 3 h. After cooling at −10°C, the resultant precipitate was filtered, washed with a small amount of cold ethanol and dried to give 17·2 mg (59% yield) of light tan powder. The specific activity was 58 mCi mmol^{-1} (0·36 mCi mg^{-1}). The melting point of the labelled product was >300°C. Purity was determined by TLC using a Bioscan BID 100 Image Analyzer, eluting with CH_2Cl_2–MeOH–NH_4OH (35:65:1). IR and NMR spectra were consistent with the cold material.

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Disposition of [^{14}C]2,3-dihydrophthalazine-1,4-dione

Intravenous administration. Sprague Dawley rats (300±6 g) received an intravenous bolus dose of 13·7 μCi of [^{14}C]2,3-dihydrophthalazine-1,4-dione (20 mg kg^{-1}) dissolved in phosphosaline buffer (PSB: 0·136 M NaCl, 0·0026 M KCl, 0·008 M Na_2HPO_4–$7H_2O$, 0·075 M KH_2PO_4), pH 7·4, into the femoral vein, and were placed in metabolism cages. Blood samples (0·3 mL), collected from the tail vein, and cumulative faecal and urinary recoveries were collected for 144 h after drug administration. Plasma was harvested at selected times after drug administration and an aliquot of plasma (50 μL) was plated on No. 1 filter paper disks, dried, quenched with hydrogen peroxide (30% volume) and counted. Faecal samples were homogenized in water (10×). Aliquots (100 μL) of urine or faecal homogenate were counted. All scintillation counting was done with a Packard Scintillation Spectrometer using an internal standard and a quenching program for the chemiluminescence of the cocktail mixture and tissue digestion medium.

Oral administration. Sprague Dawley male rats (350±6 g) received an oral dose of 13·7 μCi of [^{14}C]2,3-dihydrophthalazine-1,4-dione (20 mg kg^{-1}) dissolved in 1% carboxymethylcellulose. The rats were placed in metabolism cages and blood was collected for 240 h, while urine and faecal samples were collected for 336 h. Samples were analysed as detailed in the intravenous administration study.

Absorption of [^{14}C]2,3-dihydrophthalazine-1,4-dione from the stomach and small intestine

Sprague Dawley rats (340±8 g) were anaesthetized with

pentobarbitone (22 mg kg^{-1}) and chlorpromazine (25 mg kg^{-1}), i.p. In one group of animals a ligature was placed around the pylorus. Drug (13·7 μCi, 20 mg kg^{-1}) was given orally in 1% carboxymethylcellulose and blood was drawn from the tail vein. In the second group, a ligature was placed around the small intestine approximately at the ileum, and another ligature was placed around the pylorus. The drug was introduced into the lumen below the pylorus. Blood was drawn from the tail vein at the same times as in the disposition study.

Tissue distribution of [^{14}C]2,3-dihydrophthalazine-1,4-dione
Sprague Dawley rats (350 ± 5 g) were orally administered 13·7 μCi of ^{14}C-labelled drug (20 mg kg^{-1}) in 1% carboxymethylcellulose. At 1, 2, 6, 12, 24, 48 and 336 h after drug administration, the animals were decapitated, blood was collected and tissues were excised from the animal, weighed and homogenized in 0·025 M sucrose + 0·001 M EDTA buffer. Animal carcasses, bone and skin were digested in 30% KOH heated to 55°C for 48 h to solubilize the sample. Aliquots (100 μL) were counted.

Expired $^{14}CO_2$ from [^{14}C]2,3-dihydrophthalazine-1,4-dione
Sprague Dawley rats (300 ± 4 g) were orally administered 13·7 μCi of ^{14}C-labelled drug (20 mg kg^{-1}) in 1% carboxymethylcellulose. The animals were placed in sealed glass metabolism cages with an oxygen supply. The expiratory air was exhausted into 20 mL hyamine hydroxide to trap $^{14}CO_2$. Aliquots (500 μL) were analysed for radioactive content.

Partition coefficient of 2,3-dihydrophthalazine-1,4-dione
A partition coefficient was obtained as described by Leo et al (1971) in a water–octanol mixture (20:80). The drug was quantitated by determining the amount present in each layer by UV spectrophotometry. Standard curves showed that the plot of UV absorption versus drug concentration was linear.

Albumin binding of [^{14}C]2,3-dihydrophthalazine-1,4-dione
An activity of [^{14}C]2,3-dihydrophthalazine-1,4-dione which was proportional to the maximum d min^{-1} found in plasma in-vivo (2·6 μCi, 0·06 mol) was incubated with 4·5 mL of bovine serum albumin (20%) at 25°C. The mixture was placed in a #2 dialysis bag (MW cutoff 12 000–14 000) and dialysed at 0°C against phosphosaline-EDTA buffer for 3 days. Samples were taken from both the dialysis medium and the dialysis bag and counted.

Fibroblast uptake of [^{14}C]2,3-dihydrophthalazine-1,4-dione
Human fibroblasts B6-9 were plated on tissue culture dishes (5×10^4 cells/plate) and suspended in Minimum Essential Medium + 10% lipoprotein deficient serum for 24 h, after which fresh medium containing 50 μL of [^{14}C] drug was added to the dishes. After 5, 10, 15, 30, 60, 90, 120 and 180 min, the medium was decanted and discarded. The cells were washed 6 times with PSB, pH 7·2 and the PSB was discarded. The cells were incubated with 1 mL 0·1 M NaOH and the cells scraped off the dish and pooled. Aliquots of the cells were counted.

Urinary metabolites of [^{14}C]2,3-dihydrophthalazine-1,4-dione
Preliminary urine extraction. Urine samples from Sprague Dawley rats (300 ± 6 g) orally dosed with [^{14}C]2,3-dihydrophthalazine-1,4-dione (13·7 μCi, 20 mg kg^{-1}) were pooled from 0–6 h, 6–12 h, 12–24 h and 24–48 h after administration. Samples of the urine were adjusted to pH 5, 7 or 9 and then mixed with an equal volume of water saturated ethyl acetate which was vortexed, allowed to equilibrate for 30 min and then centrifuged for 10 min at 3500 rev min^{-1}. Aliquots of the ethyl acetate and the aqueous layer were obtained and counted.

Preliminary identification of urinary metabolites. Sprague Dawley male rats (300 ± 5 g) were orally administered [^{14}C]2,3-dihydrophthalazine-1,4-dione (13·7 μCi, 20 mg kg^{-1}). The rats were placed in metabolism cages and urine was collected for 24 h. The urine was acidified to pH 5·0 (0·1 M HCl) and extracted into ethyl acetate (1:1) five times. The organic layers were pooled and an aliquot was plated on silica gel plates which were eluted in CH_2Cl_2–MeOH–NH_4OH (35:65:1). The plates were scanned with a Bioscan BID-100 Image Analyzer and the radioactivity quantitated. R_F values were compared with the parent as well as phthalamide, phthalamic acid (Chapman et al 1984), phthalic acid, and 3-methyl, 4-methyl, and 3-methoxyaryl substituted 2,3-dihydrophthalazine-1,4-diones (Murthy et al 1986).

Results

The mean plasma concentration-time curve of [^{14}C]2,3-dihydrophthalazine-1,4-dione after intravenous administration to rats showed a multi-exponential disposition (Table 1; standard deviations were < 6% the mean value). Using the non-linear regression program NLIN of the Statistical Analysis System (SAS), the terminal $t_{\frac{1}{2}}$ was estimated to be 79·5 h. Urinary excretion of total radioactivity for 144 h accounted for 21% of the dose and faecal excretion accounted for 6%. A urinary excretion rate plot of the total radioactivity excreted showed a terminal $t_{\frac{1}{2}}$ of 72·2 h (plot not shown). As the assay in the plasma and urine samples was for total radioactivity, a $t_{\frac{1}{2}}$ of 73–80 h would be the maximum value for the compound.

Oral administration of [^{14}C]2,3-dihydrophthalazine-1,4-dione produced a maximum mean plasma concentration within 7 min (Table 1). The drug was absorbed twice as rapidly from the duodenum compared with the stomach with the maximum disappearance from the duodenum occurring between 1–4 h. The plasma concentration-time curve confirmed that [^{14}C]2,3-dihydrophthalazine-1,4-dione displays a multi-exponential disposition. The data in the terminal phase of both the plasma concentration-time curve (Table 1) and urinary excretion rate plot (Table 2; plot not shown) were very erratic. NLIN estimates of the terminal phase $t_{\frac{1}{2}}$ were 194·3 h from the plasma data and 311·7 h from the urinary data. A sigma minus plot, which would assume that urinary excretion was complete, was linear in the terminal phase ($R^2 = 0·978$), and gave a $t_{\frac{1}{2}}$ estimate of 72·2 h. Urinary excretion of total radioactivity for 336 h accounted for 35% of the dose, while 11% of the dose was excreted in the faeces.

Table 1. Mean radioactive plasma levels after intravenous and oral administration of [^{14}C]2,3-dihydrophthalazine-1,4-dione (13·7 μCi) (n = 6).

Time	Intravenous admin.	Oral admin.
5 min	—	0·09*
7 min	—	0·2
15 min	4·9	0·1
30 min	2·3	0·1
1 h	1·6	0·09
2 h	0·5	0·08
3 h	—	0·06
4 h	0·02	—
5 h	—	0·02
6 h	0·02	—
12 h	—	0·01
24 h	0·007	0·01
48 h	0·003	0·005
72 h	0·002	0·006
144 h	0·001	0·007
192 h	—	0·004
240 h	—	0·003

* Percent of dose (30, 189, 890 d min^{-1})

Table 2. Mean urine and faecal excretion of radioactivity after oral administration of [^{14}C]2,3-dihydrophthalazine-1,4-dione (13·7 μCi) (n = 6).

	Urine excretion		Faecal excretion	
Time of collection (h)	% Dose excreted	Total % dose excreted	% Dose excreted	Total % dose excreted
0·25	0·2*	0·2	0·6	0·6
0·50	0·7	0·9	—	—
1·0	1·1	2·0	0·07	0·7
3·0	2·2	4·2	0·3	1·0
5·0	2·7	6·9	0·5	1·5
8·0	4·7	11·6	0·7	2·2
24·0	13·2	24·8	0·9	3·1
48·0	2·7	27·5	1·0	4·1
72·0	2·1	29·6	0·7	4·8
96·0	1·5	31·1	1·0	5·8
120·0	1·0	32·1	1·1	6·9
144·0	0·2	32·3	1·0	7·9
168·0	0·3	32·6	0·2	8·1
192·0	0·5	33·1	0·3	8·4
216·0	0·2	33·3	0·5	8·9
240·0	0·3	33·6	0·4	9·3
264·0	0·3	33·9	0·5	9·8
288·0	0·5	34·4	0·5	10·3
312·0	0·3	34·7	0·3	10·6
336·0	0·1	34·8	0·2	10·8

* s.d. < 6% mean

After oral administration, a large quantity of radioactivity appeared in the faeces after 15 min. The activity is probably contamination of the faecal sample with saliva or urine since the time is too short for the drug to have passed through the intestine.

After oral administration, several tissues showed less than 1% of the total radioactivity at all times: brain, oesophagus, heart, kidney, large intestine, liver, lungs, spleen, and thymus. The stomach, small intestine, reproductive organs, and chyme of the internal organs had the highest radioactive content at 30 min (Table 3). The skin and carcass contained a large quantity of the radioactivity at all times. Even after 336 h, 10% of the radiolabel was found in the skin and 18% in the carcass of the rat. Distribution of the drug to the skin and carcass occurred at a rapid rate since 15% of the radioactive content was found in the skin and 13% was found in the carcass 30 min after oral administration.

Table 3. Mean tissue radioactivity after oral administration of [^{14}C]2,3-dihydrophthalazine-1,4-dione (13·7 μCi) (n = 6).

h	Stomach (%)	Sm. Int. (%)	Reprod. (%)	Skin (%)
0·5	11·4*	5·8	2·4	14·7
1·0	5·0	5·7	2·3	17·3
2·0	4·7	1·2	0·1	30·2
6·0	4·3	1·0	0·1	19·1
12·0	0·2	0·3	0·1	18·9
24·0	0·1	0·2	0·1	16·6
48·0	0·1	0·3	0·1	12·9
336·0	0·1	0·1	0·1	9·5

h	Carcass (%)	Chyme (%)	Caecum (%)	Blood (%)
0·5	13·0	48·8	0·3	1·8
1·0	12·2	48·4	0·1	1·5
2·0	22·3	11·7	2·7	1·4
6·0	29·4	1·9	8·4	0·3
12·0	27·5	0·7	1·0	0·2
24·0	19·1	0·5	0·3	0·2
48·0	19·9	0·3	0·7	0·1
336·0	17·8	—	—	—

* Percent of dose (s.d. < 6% mean)

There was no evidence that the drug was expired as $^{14}CO_2$ (0·1%). The drug proved to be highly water soluble with a partition coefficient of log P = 0·107. Protein binding studies demonstrated that less than 2% of the drug was bound to serum albumin. Fibroblast uptake of the drug reached a maximun of 0·13% within 4 h and then decreased in content within the cells after 8 h (Table 4).

Sixty-six percent of the radioactivity excreted in the urine was the parent drug (R_F 0·34). One other major metabolite (R_F 0·52) represented 27·5% of the radioactivity. The remaining 6·4% of radioactivity was dispersed throughout the plate. Blank urine mixed with the [^{14}C]2,3-dihydrophthalazine-1,4-dione and incubated for 24 h at room temperature demonstrated only the parent drug.

When aliquots of the urine were adjusted to pH 5·0, 7·0 or 9·0, and extracted with water saturated ethyl acetate, the urine at pH 5·0 showed 61·5% of the radioactivity in the organic layer and 38·5% in the aqueous layer. At pH 7·0, 4% of the radioactivity was found in the organic and 96% in the

Table 4. Fibroblast uptake of [^{14}C]2,3-dihydrophthalazine-1,4-dione (13·7 μCi) (n = 5).

Time (min)	Intracellular d min^{-1}
5	0·053 ± 0·0004*
10	0·058 ± 0·0012
15	0·063 ± 0·0009
30	0·065 ± 0·0010
60	0·073 ± 0·0015
120	0·077 ± 0·0007
240	0·131 ± 0·0007
480	0·113 ± 0·0013
720	0·129 ± 0·0005
1440	0·036 ± 0·0012

* Mean percent of dose ± s.d.

aqueous phase and at pH 9·0, 2·5% was found in the organic phase and 97·5% in the aqueous phase.

A number of hydrolytic products of 2,3-dihydrophthalazine-1,4-dione were assessed as possible metabolites including phthalic acid (R_F 0·69), phthalamic acid (R_F 0·075), phthalamide (R_F 0·69), and methyl (R_F 0·87), 4 methyl (R_F 0·86) and 3 methoxy (R_F 0·87) aryl substituted 2,3-dihydrophthalazine-1,4-dione. None of these compounds were the urinary metabolites.

Discussion

[^{14}C]2,3-dihydrophthalazine-1,4-dione demonstrated a multi-exponential disposition after intravenous administration. The complex disposition pattern was also seen after oral absorption. The compound is rapidly absorbed orally, and is absorbed predominantly from the intestine.

The tissue distribution data show why the drug has a multi-exponential disposition. The percentage of radioactivity in the vital organs was generally less than 1% throughout the 336 h study. However, the skin and carcass (muscle, fat, connective tissue) did concentrate large amounts of radioactivity at 30 min with 15% of the administered dose found in the skin and 13% in the carcass. There was an increase in ^{14}C radioactivity uptake for 2 h in the skin and in the carcass for 6 h. The drug was cleared from these tissues at a slower rate than from the vital organs; even after 336 h, 10% of the administered ^{14}C dose was found in the skin and 18% in the carcass of the rats.

Fibroblast uptake indicated that the drug probably entered the cell slowly by passive diffusion. There was no evidence that the drug accumulated in these cells against a concentration gradient that would suggest a carrier-mediated system of uptake.

The urinary metabolites after oral administration appeared to be unconjugated in nature based on the ethyl acetate extraction. The major excreted metabolite co-eluted with the parent drug. The parent drug represented 66% of the excreted [^{14}C] drug and the metabolite accounted for 28%. The remaining 6·4% of the excreted ^{14}C dose seemed to be dispersed among a number of very minor metabolites.

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