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PHARMACOKINETICS OF N-(4-HYDROXYPHENYL)-all-*trans*-RETINAMIDE IN RATS

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ABSTRACT:

The concentration of N-(4-hydroxyphenyl)-all-*trans*-retinamide (HPR) was determined in plasma and a variety of tissues from rats after an intravenous dose (5 mg/kg). The plasma concentration-time curve could be accurately described by a triexponential equation. The apparent volume of distribution of HPR was approximately 10–12 liters/kg and the terminal half-life was 12 hr. Metabolites of HPR were more abundant than intact drug in most tissues 24 hr after the iv dose. A 5-day excretion study with radiolabeled HPR revealed that less than 2% of a single iv dose (5 mg/kg) is excreted as unmetabolized HPR in urine and feces and that most of the radioactivity is eliminated in the feces. HPR was incompletely absorbed after an oral dose (10 mg/kg).

The prophylactic effects of retinoids (vitamin A and structurally related compounds) on a variety of epithelial tumors are now well documented in rodents (1–6). The possible role of retinoids in the chemoprevention of cancer in man is under intensive investigation (7, 8). In addition, retinoids are of known benefit in the clinical management of several dermatological disorders, including acne vulgaris, basal cell carcinoma, psoriasis, and keratosis follicularis (7, 9–11). Although numerous retinoids have been synthesized, relatively little information is available on the pharmacokinetics and metabolic fate of these compounds (12–15). Such information will be important in directing the design and synthesis of new retinoids that exhibit more desirable properties, such as prolonged duration of action and selective accumulation in target tissues.

Recent advances in high-performance liquid chromatography have greatly facilitated studies on the fate of retinoids in biological systems (16–18). In conjunction with UV absorbance detection and/or radiotracer techniques, HPLC can be used to rapidly resolve and measure minute amounts of retinoid without degradation or isomerization. The propensity of retinoids to decompose at relatively low temperatures has limited, although not precluded, the application of gas-liquid chromatography in the analysis of these compounds (19).

In the present study, HPLC and radiotracer methods have been used to elucidate the kinetics of N-(4-hydroxyphenyl)-all-*trans*-retinamide (HPR)[1] in a variety of rat tissues and body fluids (see Fig. 1). HPR is approximately one-third as active as all-*trans*-retinoic acid in reversing squamous metaplasia of vitamin A-deficient trachea in organ culture (20). It can delay, in a dose-dependent manner, the appearance of carcinogen-induced breast tumors in rats (20). Furthermore this retinoid exhibits low toxicity in mice (21) and rats (20).

Materials and Methods

Animals. Male, Sprague-Dawley rats were purchased from Taconic Farms (Germantown, N. Y.) and maintained on Wayne Lab-Blox (Allied Mills, Chicago, Ill.) and water *ad lib.* in wire cages.

[1] Abbreviations used are: HPR, N-(4-hydroxyphenyl)-all-*trans*-retinamide; BHT, butylated hydroxytoluene.

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HPR. HPR (m.w. 391) was generously supplied by Dr. Robert Gander, Johnson & Johnson (New Brunswick, N. J.). Purity of this retinoid was >99% as determined by HPLC and UV detection at various wavelengths. HPR was stored over liquid N_2 and was brought to room temperature in a desiccator under vacuum before exposure to air. All procedures involving the manipulation of HPR or biological samples containing HPR were performed under yellow or subdued, indirect white light in order to avoid isomerization of HPR and its metabolites. Although HPR remained stable in methanol for up to 6 months at −20°C, methanolic solutions of this amide were not employed for quantitative analyses after 1 week of storage. HPR solutions for iv infusion were freshly prepared for each experiment, whereas solutions for oral dosing were utilized for up to 5 days (stored at 4°C).

Radiolabeled HPR was synthesized by New England Nuclear (Boston, Mass.) from tritiated *p*-aminophenol (label uniformly distributed in the phenyl ring). Tritiated HPR was purified by HPLC within 1 week of each experiment to remove the antioxidant, BHT, and some minor radioactive impurities (final purity was >99%).

Plasma Pharmacokinetics. HPR (30 mg/ml in ethanol) was administered at a dose of 5 mg/kg to ether-anesthetized rats (290 ± 3 g) as a 45-sec infusion into the left external jugular vein. Other animals received HPR [13.33 mg/ml in ethanol/corn oil, 1:5 (v/v)] *via* gastric intubation at a dose of 10 mg/kg. Each rat was placed in a restraining cage which permitted access to water and regular laboratory chow. Blood samples (400 μl) were withdrawn periodically from a catheter (Dow-Corning Silastic, medical grade, 0.5 mm I.D. × 0.94 mm O.D.) that had been previously inserted into the right atrium *via* the right external jugular vein. The catheter was flushed with heparinized saline (10 units of sodium heparin per ml) after each sampling procedure. Normal blood volume was maintained during the experiment with infusions of fresh blood from donor rats. Each blood sample was immediately centrifuged for 2 min in a heparinized tube (1.5 ml, stoppered) in a Brinkmann model 3200 table-top centrifuge. Plasma (200 μl) was lyophilized in a Reactivial (5 ml, screw-capped; Pierce Chemical Co., Rockford, Ill.) and then extracted with 300 μl of methanol (Spectrograde; Burdick & Jackson Labs. Muskegon, Mich.). The methanol extract was analyzed for HPR as described below.

Distribution Studies. Each of nine rats (294 ± 10 g) received a single 45-sec infusion of tritiated HPR (specific activity, 6.4 μCi/mg) at a dose of 5 mg/kg in approximately 50 μl of ethanol into the left external jugular vein. Animals were killed at various times after the injections, and tissues were rapidly removed, weighed, frozen in containers submerged in Dry Ice/ethanol and then stored at −120°C. Other rats received HPR [15 mg/ml in ethanol/corn oil, 1:5 (v/v)], 10 mg/kg once each day by gastric intubation for 14 days. Tissues were collected from these animals at 4 and 24 hr after the last dose. Additional information on the distribution of HPR after oral administration was obtained by measuring in rats with biliary fistulas the amount of HPR and its phenyl metabolites that appear

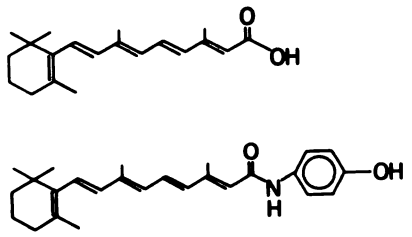

FIG. 1. *Structure of all-trans-retinoic acid (top) and HPR. (bottom).*

in bile and feces during a 24-hr period after a single dose (10 mg/kg). Each tissue was homogenized in 2 volumes of an ascorbic acid-trisodium EDTA solution (0.5 mg of each per ml of water) on ice with a Tissumizer (model SDT-182E, Tekmar, Cincinnati, Ohio). Plasma or tissue homogenate (200 μl) were lyophilized in a 5-ml Reactivial and then extracted with 300 μl of methanol. After centrifugation at 9000*g* for 8 min at 4°C, the methanol extract was subjected to HPLC analysis.

Excretion Study. Tritiated HPR (30 mg/ml in ethanol; specific activity, 5.76 μCi/mg) was infused into the left external jugular vein of rats (280 ± 9 g) at a dose of 5 mg/kg. Animals were maintained separately for 5 days in metabolism cages designed for the separation of urine and feces. The rats had free access to water and laboratory feed throughout the experiment. Urine receptacles were kept on ice and shielded from light during the collection period. Every 24 hr, the urine and feces were removed and stored at −120°C, and the collection funnel of each cage was rinsed with methanol. Fecal material was homogenized in 5 volumes of ascorbic acid-trisodium EDTA solution (0.5 mg of each per ml of water). Aliquots (200 μl) of urine and fecal homogenate were lyophilized in Reactivials and extracted with 300 μl of methanol. After centrifugation at 9000*g* for 8 min, the methanol extracts were analyzed for HPR by HPLC.

HPLC Assay. Methanol extracts (75 μl) and standard solutions of HPR were all analyzed on a Partisil PXS 10/25 ODS-2 reverse-phase column (Whatman, Clifton, N. J.) in either a Spectra-Physics model 8000 or model 3500 instrument with acetonitrile/water (4:1, v/v) as a mobile phase (flow rate, 1.2 ml/min). A Spectra-Physics model 8200 UV absorbance detector (365-nm filter) and electronic peak integrators were employed for quantifying HPR. As little as 2.5 pmol HPR could be accurately measured. Standard curves were prepared daily by extracting known amounts of HPR from tissue homogenate or plasma. Percent recovery for the extraction procedure was determined for each tissue by comparing detector response to standards with the response to extracted HPR standards.

Measurement of Radioactivity. Portions of homogenized tissue, diluted feces, intact body fluids, and methanol washes from cages were placed on cellulose pads in small paper cups (Combusto-pads and Combusto-cones, Packard Instrument Co., Downers Grove, Ill.), treated with Packard Combustaid, and burned in a Packard model B306 Tri-Carb Tissue Oxidizer. Tritiated water was collected in 15 ml of Packard Monophase-40 scintillation medium, and cpm were determined in a Packard model 3255 scintillation counter. The external standardization method was used for calculating absolute dpm. Percentage recovery from the oxidizer was ascertained for each tissue, and this ranged from 89 to 94%.

Results

Assay for HPR. The described assay for HPR was found to be fast, reproducible, and accurate. HPR was clearly resolved by HPLC from other substances that absorbed at 365 nm (fig. 2). Standard curves (ranging from 0.13 to 255 nmol/g of tissue) were linear for all tissues. The recovery of HPR during extraction ranged from 86 to 95% for the various tissues, except for adipose tissue (recovery = 63%). Introduction of other retinoids as internal standards prior to the lyophilization step did not significantly improve the accuracy or precision of the assay.

Plasma Pharmacokinetics. The plasma concentration-time curve for HPR (5 mg/kg) is shown in fig 3. A best-fit line was determined by using a three-compartment open model. The curve is characterized by an initial, pronounced decline in drug concentration (half-life = 2.4 min), a prolonged (7-hr) secondary drop, and a final, first-order decline (half-life = 12 hr). The apparent volume of distribution for HPR in the rat is estimated to be 10–12 liters/kg body weight.

The appearance of HPR in rat plasma after a single po dose (10 mg/kg) is shown in fig. 4. The peak concentration was achieved 4 hr after drug administration. Plasma levels were more variable than after iv infusion and were substantially lower than anticipated. Thus, the average area under the plasma concentration-time curve after 10 mg/kg po was only one-third as large as that after 5 mg/kg iv. In addition, the elimination half-life appeared to be shorter than after an iv infusion.

Tissue Distribution. Table 1 indicates the relative abundance of HPR and its metabolites in various tissues 1, 8, and 24 hr after an iv dose (5 mg/kg) of HPR. For calculating the percentage of dose in skin, bone, skeletal muscle, and plasma, tissue weights were based upon reported values (22, 23). The plasma concentrations of HPR were not significantly different from those reported for corresponding times in fig. 3. The most striking feature of the 1-hr distribution data is the high concentration of intact HPR in liver. Other tissues with notably high levels of HPR 1 hr after dosing are the kidneys, lungs, spleen, heart, and pancreas. By comparing distribution data at 1, 8, and 24 hr after injection, one can estimate both the rate at which various tissues achieved concentration equilibria with plasma, as well as the steady-state tissue/plasma concentration ratios (T/P). Thus, HPR equilibrated rapidly between plasma and the compartments represented by spleen, and lung. In contrast, diffusion of circulating HPR into and out of adipose tissue, brain, and testes was slow, as evidenced by continually increasing T/P for these latter tissues. Many tissues either approached or achieved equilibrium with plasma with respect to HPR concentration by 24 hr after a single iv dose. These observations are further corroborated by data on the distribution of HPR after chronic dosing (table 2). Thus, T/P values 24 hr after chronic dosing (po) are nearly the same as those 24 hr after a single iv dose. Predictably, the T/P for adipose tissue was larger after chronic dosing, indicating that HPR was retained longer in

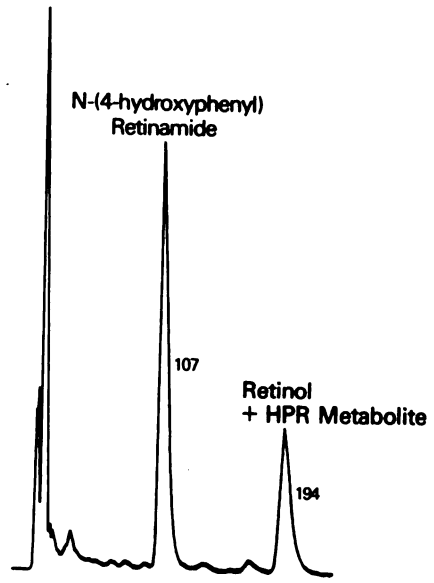

FIG. 2. *Chromatogram of a methanol extract of rat liver.*

Tissue was obtained 8 hr after 5-mg/kg dose of HPR, iv. Peak retention times are reported in tenths of minutes. The peak at 19.4 min was later found to contain endogenous retinol and a metabolite of HPR.

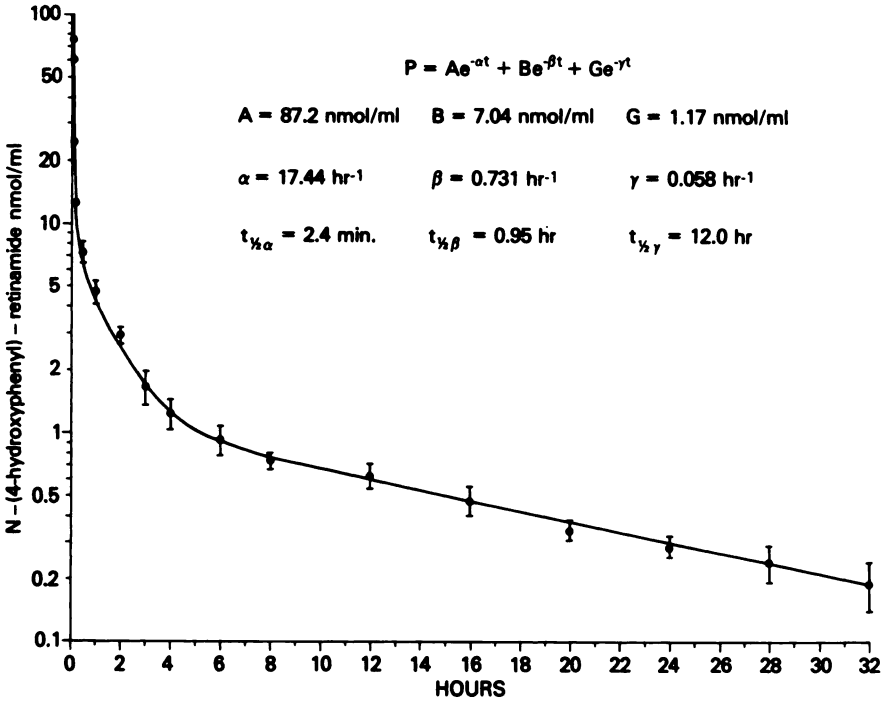

FIG. 3. *HPR concentration in plasma after a rapid iv infusion (5 mg/kg).*

Points and bars represent means ± SD for four animals. A best-fit line was obtained by fitting the data to a triexponential equation.

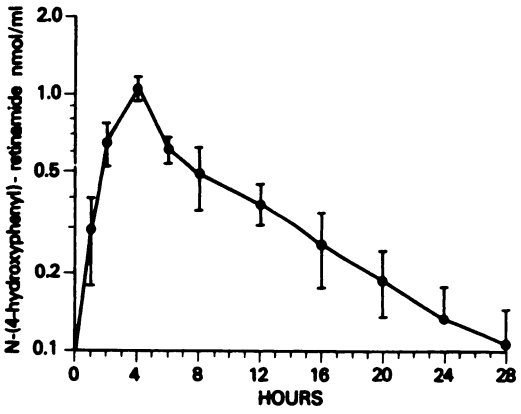

FIG. 4. *HPR concentration in plasma after 10 mg/kg, po.*

Points and bars represent means ± SD for four animals.

fat than in other tissues. Large T/P ratios for stomach, small intestine, and large intestine after oral dosing were, of course, a result of direct exposure of these organs to the drug bolus.

Table 1 further reveals that metabolites of HPR accumulate in many tissues. At 24 hr after tritiated HPR administration (5 mg/kg, iv), intact HPR constituted only one-third of the total radiolabel in most tissues.

Rats with Biliary Fistulas. When rats with biliary fistulas were given radiolabeled HPR (10 μCi; 10 mg/kg) orally, only about 40% of the dose was absorbed. This is suggested by the following observations. Twenty-four hours after dosing, 60% of the dose was located in the feces and large intestinal lumen (almost exclusively as intact HPR); 1.5% of the dose remained in the stomach and small intestine (as unchanged HPR); and 15% of the dose had been excreted into the bile, primarily as HPR metabolites.

Excretion of HPR and Radioactivity. Fig. 5 illustrates the cumulative excretion of radiolabel into urine and feces after an iv dose of tritiated HPR (5 mg/kg). The fact that intact HPR represents < 2% of the fecal and urinary radioactivity during the first 5 days indicates that biotransformation has a prominent role in the elimination of HPR from the body. Approximately 64% of the dose was excreted within 2 days and an additional 13% was discharged during the next 3 days. Thus a portion of the dose was retained for a relatively long period of time—much longer than would be predicted from the half-life of HPR (see fig. 3).

Discussion

The pharmacokinetics of HPR have now been described in the plasma and numerous tissues of the rat. The doses employed (5 mg/kg, iv, and 10 mg/kg, po) are nontoxic and are comparable to the daily doses used previously to successfully demonstrate chemoprevention of cancer in rats. Pharmacokinetic parameters that describe the initial uptake phase for HPR in plasma after an iv dose are probably of little practical importance, inasmuch as HPR is unlikely to be used iv in cancer chemoprevention studies. Also, the initial disappearance of HPR from plasma as shown in fig. 3 may be influenced by partial precipitation of this hydrophobic retinoid on plasma proteins. On the other hand, the plasma disappearance of HPR at later times probably reflects clearance of this compound by true physiological mechanisms. Therefore, parameters derived from the later time points can be employed for comparing the elimination half-life of HPR with the half-lives of other retinoids and for planning dosage schedules for HPR in future experiments. One important observation is that the pharmacokinetics of HPR in the rat appear to be substantially different from those of its closest natural analogue, all-*trans*-retinoic acid. In comparison with all-*trans*-retinoic acid, HPR exhibits a much longer elimination half-life (12 hr *vs.* 20 min) and achieves higher

TABLE 1

Distribution of HPR after an iv dose (5 mg/kg)

The mean SD (N=3) was ±14% for the various tissues.

Tissue	1 hr after Injection					8 hr after Injection					24 hr after Injection				
	HPR Only			HPR and Phenyl Metabolites		HPR Only			HPR and Phenyl Metabolites		HPR Only			HPR and Phenyl Metabolites	
	Concentration	Tissue / Plasma	% of Dose in Tissue	Concentration	% of Dose in Tissue	Concentration	Tissue / Plasma	% of Dose in Tissue	Concentration	% of Dose in Tissue	Concentration	Tissue / Plasma	% of Dose in Tissue	Concentration	% of Dose in Tissue
	nmol/g			*nmol/g*		*nmol/g*			*nmol/g*		*nmol/g*			*nmol/g*	
Plasma	5.6	1	1.8	8.7	2.8	0.90	1	0.28	3.3	1.03	0.24	1	0.08	1.9	0.60
Liver	110	20	29	140	37	11	12	2.7	34	8.3	2.5	10	0.70	14	4.0
Kidneys	35	6.3	1.9	38	2.1	19	21	1.1	26	1.5	4.6	19	0.28	15	0.87
Lungs	32	5.7	1.5	44	2.0	7.4	8.2	0.28	16	0.61	1.4	5.8	0.06	6.4	0.26
Spleen	29	5.2	0.50	51	0.88	3.9	4.3	0.07	16	0.29	1.1	4.6	0.02	7.7	0.14
Pancreas	15	2.7	0.43	20	0.57	15	17	0.43	20	0.59	2.4	10	0.07	8.3	0.23
Testes	1.1	0.20	0.10	1.9	0.17	1.1	1.2	0.10	2.7	0.23	0.77	3.2	0.07	2.5	0.23
Prostate	4.7	0.84	0.07			3.4	3.8	0.05			1.4	5.8	0.02		
Stomach	9.9	1.8	0.44	9.6	0.43	5.8	6.4	0.27	7.2	0.33	1.7	7.1	0.08	3.6	0.16
Small intestine	9.8	1.8	1.2	25	3.1	3.3	3.7	0.50	7.6	1.2	1.4	5.8	0.22	3.9	0.64
Small intestine contents			1.7		9.1			0.37		11.1			0.08		3.3
Large intestine	6.8	1.2	0.48	6.8	0.48	3.7	4.1	0.25	9.0	0.61	1.8	7.5	0.13	4.3	0.34
Large intestine contents			0.08		0.50			0.69		23			0.25		7.2
Brain	1.1	0.20	0.05	2.1	0.10	1.4	1.6	0.07	2.8	0.14	0.96	4.0	0.05	2.4	0.12
Heart	26	4.6	0.67	25	0.65	7.0	7.8	0.18	8.1	0.22	3.0	12.5	0.08	3.3	0.09
Adipose tissue	5.5	0.98	0.36	5.4	0.35	6.7	7.4	0.44	8.1	0.53	5.5	23	0.36	9.3	0.61
Bone	6.4	1.1	3.2			1.9	2.1	1.0			0.60	2.5	0.28		
Skin	3.0	0.54	4.0			1.2	1.3	1.6			0.17	0.71	0.23		
Skeletal muscle	4.8	0.86	16			4.9	5.4	17			1.7	7.1	6.0		

concentrations in tissues relative to plasma levels[2] (24). In order to maintain relatively constant levels of unmetabolized retinoid in plasma, all-*trans*-retinoic acid would have to be administered much more frequently than HPR. This factor may be important in cancer studies if retinoids must be continuously present in order to completely suppress the appearance of carcinogen-induced tumors.

A major limitation to the employment of retinol or retinyl acetate for cancer chemoprevention has been the development of liver disease when these compounds are used in high doses for prolonged periods of time (25). This toxicity appears to stem from a gradual accumulation of both retinol and retinyl esters in the liver. The current study confirms that HPR and its metabolites are not stored in the liver (even though hepatic levels of these retinoids are transiently elevated after each dose of HPR) (20). Therefore, the type of hepatotoxicity that is associated with hypervitaminosis A should not result from chronic ingestion of HPR.

The role of metabolism in the activation of retinol has been the subject of intensive investigation (26–28). All-*trans*-retinoic acid is widely recognized as a physiologically important metabolite of retinol (29);' and biologically active metabolites of retinoic acid have been described (18). Our data show that a synthetic retinoid, HPR, undergoes extensive biotransformation and that its metabolites accumulate in several tissues. The identity of these metabolites and their possible contribution to the biolgical activity of HPR is now being examined in our laboratory. The information on HPR metabolite distribution in table 1 is somewhat compromised by the intramolecular location of the tritium label employed in those experiments. It is conceivable that the amide bond in HPR is hydrolyzed *in vivo* to release radiolabelled *p*-aminophenol, which in turn may be converted to a variety of nonretinoid metabolites. However, preliminary experiments indicate that amide hydrolysis of HPR is a minor or nonexistent metabolic pathway. Our laboratory has recently obtained HPR that is tritiated in the retinoyl moiety ([10-^{3}H]HPR). Bile was collected from rats receiving [10-^{3}H]HPR alone or [*phenyl*-^{3}H]HPR alone at a dose of 5 mg/kg, iv. Biliary excretion rates for the two kinds of radiolabeled HPR were not different (cumulative excretion was about 52% on day 1 and 71% on day 2). When bile samples were analyzed by reverse-phase HPLC with acetonitrile/1% ammonium acetate gradients, it was found that 85% of the radiolabel in bile was associated with six major metabolites. All of these metabolites possessed an intact amide bond. Furthermore, < 2% of the radiolabel in bile from rats receiving [*phenyl*-^{3}H]HPR had HPLC elution times that corresponded to *p*-aminophenol and its conjugated metabolites.

Our data indicate that HPR is incompletely absorbed after oral administration. In other experiments, we found that utilization of other vehicles such as dimethyl sulfoxide or trioctanoin-ethanol did not significantly improve absorption of HPR. These observations perhaps explain the alleged lower toxicity of HPR in rodents when it is administered po rather than ip (21). Inasmuch as there is little published information on the absorption of retinoids in general, we cannot conclude that the bioavailability of HPR is less than those of other orally administered retinoids. In fact, some studies show that retinoic acid, retinal, retinol, and at least one synthetic retinoid (R010-9359) are incompletely absorbed as well (12,30).

Other workers have shown previously in rodents that HPR is nontoxic in doses up to and exceeding 200 mg/kg/day ip (21),

[2] B. N. Swanson, C. A. Frolik, D. W. Zaharevitz, P. Roller, and M. B. Sporn, submitted for publication.

TABLE 2

Distribution of HPR after chronic dosing (10 mg/kg/day for 14 days, po)

The mean SD ($N = 3$) was ±16% for the various tissues.

Tissue	4 hr after Last Dose		24 hr after Last Dose	
	Concentration	Tissue / Plasma	Concentration	Tissue / Plasma
	nmol/g		*nmol/g*	
Plasma	1.5	1	0.15	1
Liver	21	14	1.6	11
Kidneys	16	11	3.4	23
Lungs	12	8	1.2	8
Spleen	7.3	4.9	0.42	2.8
Pancreas	10	6.7	2.5	17
Testes	1.3	0.87	0.69	4.6
Prostate	3.9	2.6	1.1	7.3
Stomach	42	28	3.0	20
Small intestine	177	118	2.9	19
Large intestine	3.2	2.1	0.66	4.4
Brain	2.0	1.3	1.4	9.3
Heart	13	8.7	0.74	4.9
Adipose tissue	14	9.3	4.8	32
Bone	3.0	2	0.30	2
Skeletal muscle	3.7	2.5	0.83	5.5
	total nmol		*total nmol*	
Stomach contents	1650		36	
Small intestine contents	2030		19	
Large intestine contents	185		43	

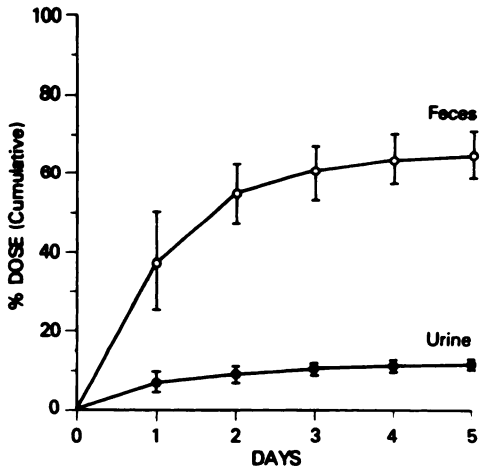

FIG. 5. *Cumulative excretion of radioactivity into urine and feces after a single iv infusion of tritiated HPR (5 mg/kg).*

As determined by the HPLC-UV assay, 1.8% of the initial dose appeared as unmetabolized HPR in feces during the 5 days. No intact HPR was detected in urine. Points and bars represent ± SD for four animals.

and that relatively low doses of this retinoid (approximately 20 mg/kg/day po) can significantly reduce the incidence of carcinogen-induced tumors (20). When young rats were given 45.6 μmol of HPR (170–313 mg/kg po daily for 12 days, with 2-day interruption between the fifth and sixth doses), no bone fractures or significant interference with weight gain were observed. Similarly, HPR had minimal or no effect on survival rate, liver function, and various hematologic values in mice when given po at a dose of 300 mg/kg/day for 21 days. These data, together with our own information on HPR kinetics in rats, indicate that the potential use of HPR in the prevention and management of cancer and dermatoses in man should be rigorously examined in future studies.

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