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Biliary Excretion of Phenol Red by Wistar and Gunn Rats[1,2] (38040)

ELTON R. HOMAN AND ANTHONY M. GUARINO

Laboratory of Toxicology, National Cancer Institute, Bethesda, Maryland 20014

The Gunn rat is a jaundiced autosomal recessive mutant of the Wistar strain, deficient in the ability to excrete bilirubin (1). This deficiency is due to the absence in homozygotes of bilirubin uridine diphosphate glucuronyl transferase (EC2.4.1.17; UDGT) (2). The Gunn rat can, however, form glucuronides from some substrates and the scope of this capability has been the subject of several investigations (2–7).

Phenol red (PR, phenolsulfonphthalein) is normally excreted largely by the kidneys in humans (8) and dogs (9) but significant amounts appear in the bile of some other species, including frog (10), chicken (11), dogfish (12) and rat (13). In the latter two species glucuronide conjugation was observed. In the present study, biliary excretion of PR and PR glucuronide were measured in Gunn and normal Wistar rats.

Materials and Methods. Phenol red was obtained from Hartman-Leddon Co., Philadelphia, PA. β-Glucuronidase (bovine liver), 12.9 Fishman Units/g, was the product of Sigma Chemical Co., St. Louis, MO. Thin layer chromatography plates, Silica Gel G, 250 μm thick, 20 × 20 cm, were purchased from Analtech, Inc., Newark, DE. Other chemicals and solvents were of reagent grade and obtained from commercial sources.

Male rats of the Wistar-Lewis strain were obtained from the Mammalian Genetics and Animal Production Section, Division of Cancer Treatment, National Cancer Institute. Homozygous and heterozygous male Gunn rats were made available by the Veterinary Resources Branch, Division of Research Services, National Institutes of Health. The rats were surgically prepared in dorsal recumbency under intraperitoneal pentobarbital anesthesia (60 mg/kg). Through a midline abdominal incision the renal pedicles were ligated and the bile duct was cannulated with a blunted 23 gauge hypodermic needle shaft attached to a polyethylene catheter (Clay-Adams PE50) 20 cm in length. Supplementary pentobarbital was dripped into the abdominal cavity as necessary. The incision was closed around the externalized catheter and body temperature was recorded and regulated through the use of rectal thermistor probes. Heat was provided by a 60 cm waterproof heating element (Briskeat, from VWR Scientific, San Francisco, CA) under the animal and a 100 W incandescent bulb above.

Bile was collected in tared 1.6 ml Eppendorf conical polyethylene vessels. Bile collections were made for thirty minutes before administration of PR and for one or three thirty minute periods after administration.

PR was dissolved in distilled water (10 mg/ml), filtered through Millipore membrane filter (0.45 μm) to remove any possible particulate material and administered via the femoral vein at a dosage level of 10 mg/kg.

At the end of the bile collection, cardiac blood was taken in a heparinized syringe, centrifuged and the plasma aspirated. An aliquot of plasma was removed for bilirubin assay. The liver was removed, weighed, and homogenized in nine volumes of absolute methanol. Methanolic extracts of bile and

[1] A preliminary communication of a portion of this data was presented before the Federation of American Societies for Experimental Biology, April, 1973, Atlantic City, NJ.

[2] To whom reprint requests should be directed.

plasma were similarly prepared. After centrifugation at 200*g* for 15 min, the supernates were stored in a refrigerator prior to subsequent procedures. Assay of direct and total bilirubin in rat plasma was carried out using Autotechnicon Model 1260 Analyzer (Autoanalyzer Method File N-12b 1/11, 1970).

Aliquots of 1 ml of the methanolic extracts of bile, serum, or liver extract were acidified with 1.0 ml of 1.2*M* HCl and nonpolar constituents extracted by addition of 1.0 ml of chloroform and agitation. The upper (polar) layer, containing free and conjugated PR, was aspirated and aliquots were placed into Eppendorf centrifuge tubes for assay of free and total PR respectively. The aliquots for assay of total PR were heated at 95° in sealed tubes for 30 min to hydrolyze the conjugated drug. Completeness of hydrolysis at this temperature and time was demonstrated by Hart and Schanker (13). The aliquots for assay of free drug were processed immediately without heating. After centrifugation at 12,000*g* for 2 min, 0.5 ml aliquots were removed and alkalinized with 0.5 ml of 2.5 *M* NaOH. After a second centrifugation at 12,000*g* for 2 min, extinction was measured promptly at 560 nm in a Gilford Model 2400 Spectrophotometer.

To test recovery, PR was added to fresh bile, serum, and homogenized liver to produce final concentrations of .50 mg/g and .05 mg/g. Recovery was 103.4 ± 4.6% (mean ± S.D.) and was similar for both concentrations and all sources. The average coefficient of variation among replicate samples was 2.5%. Heating of acidified extracts produced a 24.5% loss of optical density, necessitating separate standard curves for free and total PR. For convenience, standard curves were prepared by addition of PR to methanolic extracts of bile, liver and plasma. Subsequent treatment of standards was as previously described. The method is sensitive down to concentrations of approximately 25 μg/g of tissue or bile. All samples for PR analysis were run in triplicate. Statistical values and tests were calculated using standard procedures presented by Snedecor (21). Exponential constants were calculated from linear regression of natural logarithms of PR values on time.

Identification of the acid-hydrolyzable conjugate of PR as a glucuronide was confirmed by thin layer chromatography of acid- and β-glucuronidase hydrolysates in two solvent systems as described by Adamson and Guarino (12). Activity of the β-glucuronidase was confirmed by hydrolysis of phenolphthalein glucuronide and thin layer chromatography of the hydrolysates.

Results. Table I shows data on both direct and total plasma bilirubin levels in the two rat strains. Direct reacting bilirubin levels in Gunn rats were twice those of Wistar rats and total bilirubin levels were approximately 15 times those in the normal Wistar strain.

The observed weights of bile samples, PR concentrations, and calculated amounts of free and total PR are shown in Table II. The percentages of drug appearing as conjugated drug during each of the three periods were 33 ± 3%, 35 ± 3%, and 32 ± 4% (Wistar rats) and 2 ± 6%, 1 ± 5%, and 0 ± 3% (Gunn rats). Concentrations of PR in plasma and liver after 90 min were below the sensitivity of the method.

In a second series of experiments, Wistar and Gunn rats were sacrificed 30 min after the administration of PR in order to permit comparison of concentrations and amounts in bile, liver and plasma in the two strains. Amounts of drug in bile and liver were calculated as the product of concentration and weight; amount in plasma was calculated from concentration, body weight and an assumed plasma volume of 31.3 ml/kg (22). These results appear in Table III.

Discussion. The fraction of PR remaining

TABLE I. Serum Bilirubin Values of Wistar and Gunn Rats.[a]

Strain	Direct bilirubin (mg/100 ml)	Total bilirubin (mg/100 ml)
Wistar	0.26 ± 0.07	0.40 ± 0.10
Gunn	0.51 ± 0.15	6.20 ± 1.14

[a] Data are means and standard deviations of observations in 6–7 animals.

TABLE II. Phenol Red (PR) in Bile of Wistar and Gunn Rats.[a]

Bile collection interval	Bile Wt. (g)	Free PR		Total PR	
		Conc. (mg/g)	Amt. (mg)	Conc. (mg/g)	Amt. (mg)
		Wistar Rats			
Pretreatment	.67 ± .17				
0–30 min	.72 ± .17	1.17 ± .14	.86 ± .24	1.77 ± .26	1.29 ± .35
30–60 min	.67 ± .15	.61 ± .11	.40 ± .04	.93 ± .18	.62 ± .08
60–90 min	.59 ± .29	.36 ± .08	.20 ± .06	.53 ± .11	.29 ± .09
		Gunn Rats			
Pretreatment	.99 ± .12				
0–30 min	1.09 ± .16	1.42 ± .25	1.55 ± .36	1.53 ± .17	1.53 ± .38
30–60 min	.88 ± .26	.82 ± .10	.71 ± .14	.86 ± .04	.70 ± .13
60–90 min	.66 ± .34	.53 ± .07	.35 ± .18	.55 ± .05	.34 ± .16

[a] Data are mean ± S.D. from 5–6 animals after treatment with 10 mg/kg PR.

in the body was determined from the dose administered and the total cumulative amount of drug found in the bile at each time interval. Least squares regression analysis of first order elimination produced a decay rate of 0.018/min ($t_{½}$ = 38.5 min) for Wistar rats and 0.0077/min ($t_{½}$ = 90.6 min) for Gunn rats. The individual data points and the calculated regression lines are shown in Fig. 1.

The zero-time intercepts of the regressions are quite close, 0.818 (Wistar rats) and 0.773 (Gunn rats). The remaining 20% of the administered dose in both cases is roughly equivalent to the estimated fraction of the drug found in the liver, based on measurements made 30 min after administration. These data are consistent with an initial rapid hepatic uptake of PR and a rate limiting step in elimination subsequent to uptake. The data of Kim and Hong (9) from studies of PR excretion by nephrectomized dogs also support this model since the appearance of drug in bile was markedly delayed relative to the decrease in plasmatic concentration. Bile/plasma and bile/liver concentration ratios of free PR in Wistar rats are significantly higher than those in Gunn rats (Table III). This suggests a relative deficiency in transport of free PR by

TABLE III. Phenol Red (PR) in Bile, Plasma and Liver of Wistar and Gunn rats.[a]

Body fluid or tissue	Free PR			Total PR		
	Conc. (mg/g)	Amt. (mg)	Bile/Tissue concentration ratio	Conc. (mg/g)	Amt. (mg)	Bile/Tissue concentration ratio
			Wistar Rats			
Bile	1.40 ± .05	1.23 ± .18		2.33 ± .17	2.05 ± .24	
Plasma	.022 ± .003	.29 ± .03	63.3 ± 8.2	.024 ± .004	.32 ± .05	98.3 ± 17.9
Liver	.024 ± .005	.31 ± .06	59.1 ± 14.1	.033 ± .009	.43 ± .08	71.9 ± 14.1
			Gunn Rats			
Bile	1.28 ± .16	1.42 ± .29		1.39 ± .12	1.52 ± .30	
Plasma	.035 ± .003	.48 ± .06	35.9 ± 4.3	.038 ± .004	.52 ± .05	35.5 ± 5.7
Liver	.044 ± .004	.52 ± .08	28.6 ± 3.1	.053 ± .006	.62 ± .11	26.4 ± 2.3

[a] Data are means ± S.D. from 4 animals 30 min after treatment with 10 mg/kg PR.

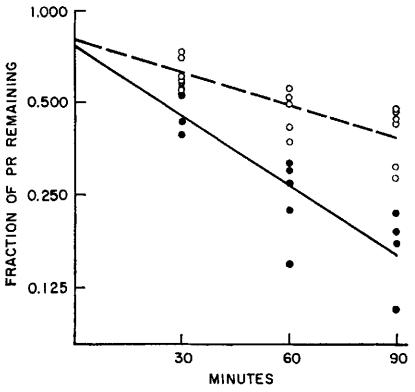

FIG. 1. Change in total body PR with time in six Wistar (○) and five Gunn (●) rats. Total body PR was calculated from dose administered less cumulative biliary excretion. The lines represent least squares regression analyses of log PR on time in Wistar (solid line) and Gunn rats (broken line).

Gunn rat liver which has not been previously noted.

An increased bile flow during the first 30 min after administration of PR to both Gunn and Wistar rats suggests a choleretic effect of PR. This effect was also noted by Sperber in chickens (14).

Phenoldibromphthalein glucuronide conjugation was reduced 10% compared to normal rats (15). No glucuronides were formed by Gunn rats from phenoltetrachlorphthein (4,5,6,7-tetrachloro-3,3-bis-(4-hydroxyphenyl)-phthalid) or bromo- or iodo- analogs in contradistinction to Wistar rats (16). However, the non-halogenated parent compound, phenolphthalein, was excreted by both strains as a glucuronide.

Renal clearances of PR in the dog and man less than would be anticipated have been noted by several investigators (17–20). Biliary excretion and/or glucuronide conjugation may constitute bases for these observations.

Summary. Biliary phenol red (PR) excretion was measured in renal ligated male Wistar and homozygous Gunn strain rats. The amount of free PR excreted in the bile was similar in the two strains. Wistar rats excreted 32%–35% of PR as a glucuronide but only free PR was found in Gunn rat bile. Bile/plasma and bile/liver concentration ratios of free PR in Wistar rats are higher than those in Gunn rats. PR exhibits a mild choleretic effect in both strains.

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